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I N D E X

Workshop on Molecular Methods in Immunohematology
Monday, September 25, 2006

I N D E X (Continued)

Page

The Americas Experience

KEYNOTE: "---" Indicates inaudible in transcript.

Welcoming Remarks

by Dr. Jay S. Epstein

DR. EPSTEIN: Good morning everyone. I'm Jay Epstein. I'm Director of the Office of Blood Research and Review at FDA's Center for Biologics, Evaluation and Research, and it is my great pleasure and privilege to invite you to this workshop on Molecular Methods in Immunohematology, which is a cosponsored effort by the FDA, the NIH National Heart and Blood Institute and the Department of Health and Human Services.

So, first of all, we are very pleased at this strong attendance. There has been a great interest in this meeting, and I think it reflects our understanding of the importance of the scientific threshold that we are on in terms of introducing greater use of molecular methods in immunohematology.

We all know that the blood grouping by serology has been an enormously successful technology which has made transfusion safe and which, I would say, doctors largely take for granted. But the experts in the field are well aware that blood incompatibility remains a significant problem in transfusion medicine and that these problems reflect certain inherent limitations of hemagglutination based testing. These include the weak reactivity of certain clinically significant antibodies, weak expression of some red cell antigens, the lack of universal test methods for antibody detection and identification and the subjective nature of the tests performed. Additionally, there a number of issues related to reagents themselves, and these also are expressed as technology limitations and they include, of course, the variability in the reagents, the lack of reagent grade antibodies and the different reactivity of monoclonal antibodies

compared to polyclonal antibodies, all of which Sheryl Kochman will elaborate upon.

But fortunately, we have the potential of a dramatic new technology, which is DNA analysis, which has the strong potential to advance this field and is already being used both in the U.S. and perhaps more extensively in Europe. DNA based analysis has been used to confirm of RHD gene, manage availability and use of rare blood types, to genotype multi transfused recipients, to resolve AVO and RHD discrepancies, to identify RHD variants that are at risk for anti-D alloimmunization, to determine RHD zygosity, to confirm the true genotype when an antigen is weakly expressed, mass screening for antigen negative and rare red cell phenotypes, genotyping donors for antibody identification panels and genotype transfusion dependent patients facilitating selection of units, all of which are known to those in this group.

Now, in this program we have a very ambitious agenda which will cover really all of these domains in a critical way. But fortunately, we will be privileged to hear from a very distinguished panel of international and U.S. national experts in this field. And also, we will hear from FDA speakers about the regulatory pathways for bringing new products to market.

The overarching goal of the workshop is to determine the potential application of molecular methods to improve safety in transfusion medicine by overcoming the limitations in the field of immunohematology to which I alluded. FDA has the additional objective to gather the information that we will need in order to chart the regulatory path forward.

So, I would just like to take a moment to extend a special thanks to

Sheryl Kochman in our staff in Office of Blood who has been the principal organizer of the workshop and your conference chairman today. And also, to Marion Reid, who played a very large role in working with the scientific program committee in helping us to bring a very excellent set of speakers here.

So, with that, I will turn the program over to the chairperson and wish everyone a successful workshop. Thank you very much.

(Applause.)

Administrative Issues and Current Problems in Immunohematology and Goals of this Workshop

By Sheryl A. Kochman

MS. KOCHMAN: Thank you, Jay. There are a few administrative details that I want to go over before we get things started. I wanted to give my acknowledgment and thanks to all of the speakers. Some of them were brought on with very little time left for them to prepare, and I especially appreciate their willingness to get things together under such extreme circumstances.

As Dr. Epstein mentioned, I would like to thank our sponsors. Dr. Jerry Holmberg and Dr. George Nemo at NIH. The planning committee consisted of Louis Barbosa,

Dr. Jonathan Goldsmith, myself, Marion Reid and Maria Rios, of CBER. We had a lot of administrative assistance in the form of getting travelers here and preparing the workshop packet and taking care of details from Rhonda Dawson, Katherine Fayne, Stacey Long, Jennifer Sharpe and Renee Wilson.

(Slide)

Some important information as it pertains to this room. There are no food or beverages allowed in this auditorium. We would like you to disable the ringer on your cell phones, pagers and PDAs. If you put it on vibrate, please careful. If you set on the counter, sometimes that vibration comes across as noise.

There is a phone at the reception desk. The phone number there is 301-496-4062. That is for incoming messages and for outgoing local calls. There is a coat room directly behind this auditorium, and if you haven't already found them, the restrooms are on the left after you exit the auditorium. There are on either side of the elevators.

Our breaks will be in the lobby area. Lunch will be in the cafeteria downstairs or, if it is too crowded there, you can cross Center Drive to go to Natcher Auditorium.

We will take questions from the floor, but if you have questions, please use your microphone and state your name and affiliation. To use the microphone that is at your particular seat you press the m-i-c button to be able to speak. We are recording this to have it transcribed so that we will have an accurate reflection of the meeting, which is one of the reasons that we ask you to identify yourself.

And for anyone who needs to come and go, there is an NIH shuttle that comes to the lobby of this building every 10 minutes and it does go to the Metro Medical Center Station.

(Slide)

And as Jay mentioned, the goals and aims of this workshop are to briefly go over some of the current problems in immunohemalogic testing.

Specifically, we have the lack of reagent grade antibodies, and the ones that FDA is most aware of are Doa, Dob, Cw, S, Fyb, Jka, Jkb and antibodies to Rh variants. We realize that this leads difficulty in patient and donor phenotyping, and it also causes some problems with characterization of reagent red bed blood cells for antibody detection and identification.

We are also aware of the variability of reactivity of monoclonal antibodies as compared to each other, ad well as the polyclonal antibodies. Our specific knowledge of this pertains to anti-D monoclonals and anti-e monoclonals. We are hearing more about problems with some of those.

We have other concerns because we are getting lots of reports of problems related to weaker activity of clinically significant antibodies, especially anti-E, anti-K, anti-Jka and anti-Jkb. We are aware of failures to detect and identify these antibodies. One concern is that if you don't detect them and identify them in the initial stages, it may allow a patient to qualify for the electronic crossmatch erroneously, which means they won't have a serologic crossmatch performed, and we also recognize that the serologic crossmatch may not detect all incompatibilities.

We are, in fact, aware of some fatalities related to these antibodies.

(Slide)

Again, in relation to the weak antibodies we have corresponding weak expression of antigens, particularly FyB, Jka and Jkb. In some cases it is because suppressor genes. In some cases it is because the true genotype and perceived phenotype don't actually match.

One of the problems that has been brought to -- well, everyone has

known for a long time that there is really not a universal single test method for performing immunohematology tests. Different methods are optimum for different antibodies, which leads to people having to have sort of a cookbook of the tests available to them, and we realize that no single method currently will detect all antibodies optimally.

And as always, we are repeatedly aware of the apparent limitations in the hemagglutination test. There are limits of detection. There are points at which the antibodies and antigens exist. We know they are there. We just can't find them. There is the subjective nature of the test performance and the reading and interpretation.

In FDA speak, these tests are normally a single 'analyte' per test kind of a test. When you do screening, you are normally testing each sample with one antibody at one time. So, you are getting one result per sample, ant sometimes you can't always automate these test methods.

(Slide)

We want to look for potential advantages of using the molecular methods in donor screening. Can we get more than one result per test? Can we automate these things?

We know that there are possibly advantages of using molecular methods in patient typing when the red blood cells or the platelets are sensitized with antibodies or when there are multiple cell populations in someone who has received multiple transfusions.

We are aware of the molecular methods being used to resolve unusual serologic findings to assist in the management of rare donor units, and in

some cases to determine a more rational approach to transfusion practices involving multi-transfused and transfusion dependent patients, those being sickle cell anemia, thrombocytopenina, thalassimia and I would add oncology patients.

(Slide)

We have also heard some interest expressed in the possibility of reagent red blood cell manufacturers using molecular methods to provide probable genotype information on the red cell donors when antisera are not available to determine the phenotype. For example, Doa and Dob. And also, to assist in the selection of homozygous cells to increase the chance of detecting weak, but clinically significant antibodies, particularly for Jka, Jkb and possibly also Fyb.

We are going to hear some of the potential use of phage display technology, identify other possible antibody detection systems, other than using the red cell, and would like to possibly be able to talk about requirements that may have to put into play; some issues that need to be resolved before we can move forward to bringing these tests to market to identify the pitfalls and limitations of molecular methods.

Having limitations does not mean FDA let the product come to market. It just means we want to know what those limitations are so that we can more adequately describe their use to the users, and we want to determine industries' -- I've put industries', plural, views of the technology because we want feedback from IBD manufacturers, we want feedback from the user community, and I consider those both industries.

(Slide)

Again, the main goal is to provide FDA with sufficient information to

begin dialog with manufacturers wishing to proceed to market, to identify potential issues of importance for the manufacturers and to identify potential issues of importance for users of molecular methods. I have to admit that in preparing this workshop and talking with people and getting things together there were even more questions than I thought there would be.

For example, will we make use of molecular methods mandatory or will it be voluntary? Will the usage be different in a blood establishment versus a transfusion service versus a reference laboratory? And so, I'm afraid there are a lot more questions than there are answer, but maybe we can get at least some of those answers at the workshop today.

With that, I would like to introduce our first speaker, Dr. Neil Avent. I'm sorry. I'm out of order. My mistake. The first speaker is going to be Christine Lomas-Francis. I jumped over her and that is a great disservice to her because she is one of the people who has put a talk together with very little time.

She is going to over some of the work of the ISBT/ICSH workshops on molecular methods in genotyping.

ISBT/ICSH International Workshops and Proficiency Test on Molecular Blood Group Genotyping

By Christine Lomas-Francis

MS. LOMAS-FRANCIS: Well, good morning. I would like to start by thanking the organizers for inviting me to participate in this meeting on this beautiful campus. I think perhaps we should all move here.

The international workshops and proficiency test on molecular blood group genotyping came into being a few years ago and are being organized under

the umbrella of the International Society for Blood Transfusion and the International Council for Standards in Hematology. It came into being at a time when more labs were begin to use DNA analysis to test for blood groups, and at that time we realized that there basically was no regulatory body for this type of testing, which also resulted in a lack of quality assurance and proficiency exercises.

(Slide)

The talk is part historical and part from the current workshop, and Marion Read was very much instrumental in helping put together these slides for the first workshop. And so, I am going to talk about the 2004 workshop, the 2005 quality assurance exercise and then bring you right up to date with the most recent workshop that took place earlier this month.

(Slide)

As I already said in the beginning, a need was identified for workshops for people who are interested in molecular analysis for blood groups, and the organizers that put this together were basically Geoff Daniels, Martin Olsson and Ellen van der Schoot. They canvassed various colleagues and friends to determine what sort of interests there would be in participation in workshops, and it was decided then to go ahead.

And for the first workshop it was also decided that a handling fee would need to be charged.

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In the 2004 workshop 40 laboratories participated and six samples were available for distribution, two of which represented DNA from transfusion dependent patients for testing for multiple polymorphisms, two represented fetal

DNA prepared from amniotic fluid and they were to be tested for rhD, rhc and K and another two samples represented plasma from RhD-negative pregnant women for fetal RhD testing.

And in both samples there were definite requests for testing to be performed for the multiply transfused patient samples. It was basically treat this as you would a sample that you received in your lab. So, the amount of testing that was done by the different participants, of course, varied.

At the feedback meeting, which took place in Edinburgh, Scotland, there were 20 laboratories who were able to send representatives.

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Jeff Daniels and his team collated all of the results, and much of the information you can see on the website at www.blood.co.uk/rsc, and much of this information came from that website. And there was also a paper that was published in 2005 in Vox Sang.

So, various different techniques were used by the participants to test for the same polymorphism. And, yes, there were some errors, but actually, it really doesn't look that bad. Most of them were within the Rh system, and several errors were very clearly clerical.

Others, in fact, were due to not testing for silencing SNPs. In particular, those that silenced the expression of the D and Dy antigens on the red cells.

(Slide)

From this workshop the participants made some recommendations. Many of them were concerning the use of controls, and not surprisingly, it was

suggested that appropriate controls should be included in the testing. Controls such as a no-template or water control to check the contamination and, of course, appropriate negative and positive controls.

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Further, the recommendations were that when testing for RhD, the tests should include for the RhD pseudo gene to check the silencing, and that new test at nucleotide 48 in Rh/ce to determine C and c antigen status was not sufficient. Duffy testing should include a test for the silencing mutation in the GATA box. And when determining RhD zygosity, it was recommended that testing should involve a method that determines the quantity of RhD genes present relative to a gene of known zygosity and should include a test for the RHD pseudo gene.

When testing maternal plasma samples, when paternally derived fetal marker is detected in tests on maternal plasma, a fetal RhD negative result should be reported with a caveat that it had not been possible to include all appropriate controls.

Furthermore, it was suggested that a numerical nomenclature should be considered for reporting blood group antigens and alleles, such as KEL1 and KEL2 instead of K and k, mainly to avoid simple reporting errors.

(Slide)

At the end of the meeting it was agreed that the workshop was a very useful exercise and that workshops will take place every two years with a feedback meeting at the ISBT Congress. The next workshop of similar format will be and, of course, was organized in 2006 again by Jeff Daniels, Ellen Van Der Schoot and Martin Olsson, and the feedback review was to be in Cape Town in September of

2006 and organized locally there by Elizabeth Smart.

In addition, in order to provide an annual external quality assurance scheme, a more limited exercise involving two blood or DNA samples and no feedback meeting will take place in the intervening years. The first one of those was organized in 2005 by Marion Reid at the New York Blood Center.

(Slide)

And this quality assurance exercise consisted of 29 participants and a fee was charged to be a part of it, but the participating laboratories did pay for the shipment of the samples. The DNA samples were from two donors who had been fully phenotyped, and they were tested for clinically relevant SNPs. And all of the results were, again, collated by Jeff Daniels and his team.

(Slide)

At this exercise a total of 496 tests were performed for polymorphisms within the ABO, MN, Ss, RhD, Kel Fy, Kidd and Dob blood groups systems, and there were only three incorrect results. In fact, in the first two the difference in Ss and MN were probably reporting errors and not test errors, but they were still errors.

And there was one determination where somebody identified a weak D instead of a D positive. And again, I advise you to go to the website if you would like more details on this.

(Slide)

The 2006 international workshop has just taken place, and so what I am presenting to you at this time is strictly from my notes. And had I known at the time I would be speaking, I may have paid even more attention than I did.

At this workshop it was truly international. There were 41 laboratories that participated and, once again, six samples were distributed. Two, as before, represented DNA from transfusion dependent patients and these samples were tested by 38 of the labs. The next two samples were, again, fetal DNA prepared from amniotic fluid for RhD, c and K testing, and these were tested by 39 laboratories.

The plasma from RhD-negative pregnant women, those two samples were tested by only 20 labs. This is still a technique very much in development. Many of the laboratories were represented at the meeting in Cape Town and in South Africa. And although, as I say, I'm talking from my notes, the final results will be published again on the website, and I believe there will be a publication in books.

The participants, when they sent in their results on the spreadsheets, there was also an additional form or questionnaire that the participants completed, and a lot of the information that was presented at the meeting is as a result of people completing those questionnaires, because it really gave a good oversight of what people were doing.

(Slide)

So again, we found that a variety of techniques were used to test for the same polymorphisms by the different labs. And, yes, there again were errors. In particular, sample one had most of the errors and most of those were within the Rh. This may be partly because the Rh was perhaps a little more complicated than first anticipated, but there was another reason also.

We had a lot more labs participating in this workshop than in the

pervious one. And secondly, when looking at the results, there were two labs that actually contributed to the majority of the errors that were reported. And so, if you take those two labs out of the mix, then the error mix would look much more pleasant to review. Again, several of the errors were clerical.

(Slide)

Analyzing the different techniques used by the participants, as I said, the range was even greater than in 2004, and they included multiplex PCR, PCR-RFLP, allele specific, real-time PCR pyrosequencing and some microarray testing. It was very obvious that most labs have a favorite method that they try to use for most things.

One of those was multiplex PCR for RHD gene determination, and while the majority of labs initially tested to see if exons 4 and 7 of RHD were present, frequently all RHD specific exons were tested for and the type of testing that was performed depended very much on the question that was being asked with the particular sample.

A trend was identified; that more labs are going towards using real-time PCR for their testing. The origin of the techniques used by the participating labs were several. Many were derived from literature. Some were developed in-house and others were commercial kits and usually a combination of any of these techniques were used.

(Slide)

I had outlined that there were recommendations from the 2004 workshop, and so the organizers reviewed to see if any of those recommendations had been implemented by the participants for this workshops, and indeed most

labs now have included appropriate controls, like the new template water control, positive and negative controls to make sure that the tests are working properly.

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Detection of the RHD pseudo gene was now being performed by most of the labs and all laboratories who ran testing for C and c used the polymorphism in RHCE intron 2 to determine C/c antigen status. Similarly, most labs were now testing for the silencing mutation for Duffy.

But when it comes to controls for the fetal DNA testing in maternal plasma, 96 labs mentioned the use of controls for the presence of fetal DNA. And numerical nomenclature, at least in the reporting that was presented at the meeting, has been implemented by some labs, but by no means all of them.

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Quite a large part of the discussion centered around the plasma samples and that represented maternal samples with the fetus being at about week 16 to 18 in gestation. One sample was an RhD-positive fetus. The other one was an RhD-negative fetus, and both of them were male.

Nineteen of the 20 labs obtained the correct result. Fifteen of those labs tested for the presence of the Y chromosome /RHD determination, which in this case would have confirmed that what they were testing was indeed fetal DNA. And the overall consensus was that the samples for the next workshops should be a little more difficult.

And from the questionnaires of these 20 labs, 15 now offer RHD determination as a routine test to their clients, only five offer RHC/c determination and three offered RHE/e and KEL1/2 determination. And the overall consensus I

think was that the test for KEL is a difficult assay.

(Slide)

There was discussion about the quantity of plasma used for this testing. Quite a range was noted. And the reason that there was discussion about this was that it was felt some of the smaller quantities being used of 100 or 200 micro liters may not be sufficient to give a sensitive enough test because the DNA extraction methods, or the manual or kit, do result in some loss of DNA. And so, there was concern that there wouldn't be sufficient DNA in the test system to detect the actual fetal DNA.

So, a need was identified for continued development for tests that can be used for samples with low levels of DNA, and in the future there was also a need identified for standardization. Both for the plasma volume used, as I have already said, and also for the particular DNA isolation method to be used.

Furthermore, standards needs to be set in place to control the effectiveness of the extraction and also the quantity of DAN that is yielded.

(Slide)

Overall the discussion points from the meeting again for everyone concerned standards and standardization. First of all, terminology for reporting the genotyping results and alleles. There were as many versions in the reports as there were participating labs.

And the terminology for alleles is now being addressed by the ISBT Terminology Committee, who happened to meet just after this particular meeting.

Again, there was a lot of discussion about genomic standards. Half of the participants used in-house controls and the experience was discussed with

regard to CE-marked standards that are now available for HLA assays, and these are from cultured cell lines. And it was suggested that once we have defined what we want as in our normal standards that perhaps cell lines could similarly be established for the red cell antigens.

When it comes to testing the more unusual variants, a sharing of resources between the different labs may be what is required, and there is a workshop by the National Institute for Biological Standards in England in March that is going to focus on some of these issues. But it really is up to the users, in the first place, to determine what it is that we need in our various panels and controls.

(Slide)

With regard to the intervening quality assurance exercise for 2007, it was decided that those wishing to partake in the external quality assurance for SNP testing could subscribe to the exercise that is being prepared for the Germany Society for Transfusion Medicine and Immunohematology. This organization has been having such an exercise since 1998 and has just contracted with an experienced non-profit organization in Germany to produce samples for these exercises. And there is a possibility of two exercises per year at the cost of 30 Euros.

It was recommended that those labs wishing plasma samples for fetal DNA can subscribe to the European Safe Testing Workshops.

(Slide)

With regard to future workshops, there was an overwhelming agreement that there should be a third ISBT workshop in 2008. There was discussion thought for this to apply a strict deadline for receipt of results if labs are

going to be using this workshops as an EQA and not to let people sneak in at the last minute. But before we can apply deadlines there are always some glitches in communication of the information that need to be ironed out.

There was discussion about the level of difficulty and the type of sample that should be sent out, and it is my understanding that what is likely to happen is that a regular sample will be sent out. By regular, something like a patient requiring multiple transfusions and that this would serve as the EQA exercise. And in addition, there would be some brain-teaser samples sent out with which the participating labs could basically use their whole repatoire of tests and then come back and brainstorm and discuss at the meeting.

And again, the plasma samples were to be included for cell-free DNA testing.

(Slide)

So, just to sum up the purpose of these workshops, they are to provide external quality assurance for laboratories that provide a diagnostic molecular blood group genotyping service, and they serve as a means of communication between those laboratories worldwide.

The workshops will continue to be held every two years with a feedback meeting at the ISBT International Congress. A smaller Q&A exercise with no feedback meeting will take place during the interim years.

And for those of you interested in these workshops, there are quite a few people here who attended both of these workshops. So, I am sure you will have an opportunity to talk with them during the break. Thank you very much for your attention.

(Applause.)

MS. KOCHMAN: I forgot to mention that in the interest of keeping to our agenda we would like to save questions for a question and answer session that follows subsets of the talks. We do have to be out of here today pretty close to 5:00. So, I do need to try and keep people on schedule.

And now, our next speaker is Dr. Neil Avent. He is Professor of Applied Science and Director of the Center for Research in Biomedicine at the University of the West of England in Bristol. He is going to speak to us about the BloodGen Project.

The BloodGen Project

By Dr. Neil Avent

DR. AVENT: Many thanks, Sheryl, and many thanks to the organizers for inviting me to talk about the BloodGen Project, which has consumed a large amount of my time and certainly the consortium's time in the last three years. Our project is about to end on the 30th of September, and I am really representing the entire consortium.

I am going to give you a few more details on what the consortium is composed of, but please, if you want further details from my talk today, go to our website and look at the further details of the project.

So, the last three years we have spent doing this and it is funded by Framework Five of the European Union; $2.5 million euros or thereabouts funding to look at methods of high throughput genotyping, and the methods that we have largely chosen is the gene chip.

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This isn't in your handouts, but I always think that this is a pretty useful description of the way that the BloodGen Project has evolved, and this first quote is from Arthur C. Clarke. I think it is about the Apollo Moon Mission back in the '60s.

"About 10 years ago I think blood group genotyping was regarded by everyone as completely impossible. Now I think probably the overall feeling is maybe it is possible, but maybe it is too expensive. But I think gradually, as we have evolved in the project, I think we are coming towards the conclusion that it is a very good idea to do mass scale blood group genotyping."

And I think Arthur's comments came from this original quote from Arthur, and I won't say the surname, about 100 years ago. So I think we are approaching between two and three.

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Now, I don't need to tell you members in the room of the drawbacks of conventional blood group serology. I think as a high technology dependent society that we live in a lot of people are fairly surprised that we are still practicing a 100-year-old technology.

We know of the imitations available with that particular technique, as we have heard already from Sheryl and Christine. Some specificities are not available or limited and certainly through the European Union serology is done routinely for those blood group systems there. But there still is a significant amount of alloimmunisation. It was on the basis of that we decided to draw the BloodGen consortium to get together to reach a mass scale genotyping platform.

(Slide)

Now, this is from the ISBT working consortium on blood group terminology. There are 29 blood group systems, of which these are the number of antigens assigned to each system, but this doesn't actually fill in the number of alleles from each system. ABO up here. Only four blood group antigens is the most clinically significant.

So, we have a very complex situation that gives rise to blood groups. We have 29 blood group systems.

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Now, the majority of blood groups have been defined at the level of the gene and many of them are a gene to blood group SNPs or pseudo nucleotide polymorphisms. And at the in the BloodGen consortium we decided that we were going to analyze those particular blood groups and the SNPs and polymorphisms that were associated with them. That is what we were going to study and have in our assessment platform.

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So most of them are simple to do. Most of the molecular genetics for those that are trained in molecular genetics are relatively straightforward. We have heard of problems with K and k, but those are caused by SNPs and it should be relatively straightforward using a genotyping platform.

But a lot of them aren't that simple. Particularly ABO and RH, and I am going to have a little aside to explain in particular why RH is much more complex.

(Slide)

Now, we have at least three major ways of being rhesus negative. In

Caucasians we have a complete RhD deletions and in Africans we have a pseudo gene where there is a virtually intact RhD gene with missense and nonsense mutations and a small duplication. And we have a hybrid RH gene in the R-prime-S phenotypes.

So, just by looking at an African and a Caucasian populations, there are multiple ways of being rhesus negative.

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And for further information, if you look at Willy Flegel's website, the rhesus base website, those are fully cataloged. If you type in Flegel rhesus base in Google, then you will come up with Willy's site as a top hit, and you can see the level of complexity that there is in the RH system.

So really, if we are going to have a genotyping platform which is going to be functional, we are going to need to address the very complex RH system as a matter of course.

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Now, of course, it is not just SNPs. In RH we have hybrid RH genes and many of us in this audience have actually spent a great deal of time characterizing these at the macro level. We have a series of hybrid RH genes that give rise to these partial D phenotypes, which we all know are clinically significant, and also, SNPs in the RhD gene can give rise to partial D phenotypes as well. We have got to be able to detect the presence of these hybrid genes, as well as single nucleotide polymorphisms in D.

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Through them all we have weak D phenotypes, and this very briefly

gives a description of what weak D phenotypes involve. This is a three-dimensional representation of the RhD monomer, and a lot of the weak D mutations are found in transmembrane domains, which affects the assembly of the proposed Rh trimer so that we get weakened D antigen expression.

So, we are beginning to understand the molecular basis of why Rh is such a complicated beast to deal with. So, if you are going to have a testing platform, you have to address those RhD alleles which are clinically important.

(Slide)

BloodGen itself was launched six years ago at the First International Workshop on Platelet and Granulocyte on Red Cell Immunology in Amsterdam. It was at that dinner at the West Indies Schoose in Amsterdam that myself, Willy Flegel, Ellen van der Schoots and Anna Rybera discussed the potential of applying for European funding.

In the ensuing next two years we got European funding and set up the BloodGen Consortium. Many of the members you have heard in talks already this morning, but those are the members.

The platform that we were going to develop was based and developed largely by Progenika, a post-genomics company based in northern Spain, the Basque area of Spain. So that was the set up the consortium. This has led up to the blood chip product that will be made by Progenika and will be launched next year.

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That was the aim. There was a demonstration project. As I said, we had $2.5 million euros or thereabouts. Progenika funded a million euros itself to

check the user validated system for high throughput group blood genotype.

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And why did we want to do this? We have heard already. Sheryl has already outlined the reasons why we should have a genotyping approach as well to support serology. If we look at haemovigilance systems, which in Europe there are going to be a standardized European haemovigilent system, we can see that a series of transfusion related incidents are reported.

In the French system, which is mandatory, there are far more transfusion related events than there is in the good old British voluntary system, which saw just 10 incidents over the same number of cases. So this still is a problem. In particular, anti-Jka causes delayed hemolytic transfusion reactions. That is certainly one of the major causes of transfusion incidents in the UK.

Now, our argument was that mass-scale genotyping could reduce if not eliminate those transfusion reactions or alloimmunisation events. And, of course, is shutting the barn door when the horse has bolted. We are not really delaying primary alloimmunisation events by crossmatching.

(Slide)

So again, what are the advantages of genotyping over serological testing? We have heard already the range of antisera is not complete. Some of these are expensive. We have red blood cells which are coated with antibodies, and therefore, are difficult to detect.

Some of them are in short supply. They are polyclonal reagents. There is variation between monoclonal reagents. They are poorly standardized. We have heard already about weak D and Fyb. And again, we have also heard

that they are difficult to alternate.

(Slide)

But there are some disadvantages of genotyping. Genotyping, at the moment, is inherently expensive. Genotype is not always phenotype and that is mainly because there are genetic variants out there that remain to be discovered. And, of course, ABO serology is very cheap and, more importantly, extremely accurate. So that is always going to be a problem. We are going to need to do genotyping. If we are going to do genotyping for ABO, it has got to compete with something which is extremely robust.

(Slide)

Microarrays have been around in press in blood grouping since about May of 2005, and we can see a few papers there from that May issue of Transfusion. So, our approach is new. Certainly members of the audience have been involved in the development of array platforms, and this is a very clear way of going for a mass-scale genotyping.

(Slide)

Now, to turn to our BloodChip Project, eventually we described it as four different packages, one to four, and the first assessment was the development of a single nucleotide polymorphism list or the alleles that we wish to detect on the platform. That was the first deliverable of the project done within six months of the project.

Then we had to simultaneously optimize DNA extraction, and we now know that DNA quality is very important to the success of the procedure. So, an optimization of DNA extraction, and our standard procedure is using the Qiagen kit.

We have to maintain the quality of DNA and a match of DNA.

Then a large amount of the efforts in the project went into the development of multiplex PCRs, which I am going to describe a little bit about the chemistry of those multiplexes, and they were targeted at those particular blood group specific genes.

And in work package four we assembled a very unique biobank of genomic DNAs collected throughout the consortium, and they were organized by Will Flegel, our next speaker, and that is held in Ulm, Germany. So, we think we have a very unique collection of rare genomic DNAs in Europe.

And the development of the chip was carried out by Progenika, and this is version four of the chip. They have 116 single nucleotide polymorphisms. Initially we started with 90. We collected a few more along the subsequent years, and we will talk a little bit about the chip platform in a moment.

(Slide)

So, this is what the chip is currently able to discriminate. It is able to discriminate. You can see a large number of the SNPs are RhD, and we are able to define 87 different genotypes for RhD and those number of alleles for the remainder. And we are also developing HPA alleles on the chip as well. So we are looking at it becoming a very comprehensive testing platform for blood groups, including platelet antigens.

(Slide)

And a little bit about the development of the multiplex PCR because this is certainly, I think, the power of the technology, and this entails the amplification of various SNPs. As I said they have got 116 SNPs on this testing

platform using gene specific primers and included in the mix are a set of primers that have tags. So, these red tags are --- tags which are a common consensus DNA sequence.

And once they are amplified with the consensus primers, then you get a very, very consistent multiplex amplification and you can amplify 20 to 30 PCR products in one chip.

(Slide)

So those 20 to 30 products are then fragmented and labeled and denatured and then those labeled products are then hybridized to the blood chip, to the array. And in order to get very strong hybridization we have a perfect match or you get a very bad hybridization, making an imperfect match. That is on the basis of the detection of the signals that we get from the chip.

(Slide)

So, to summarize the procedure, we take blood. We extract it with a standardized genomic DNA extraction method. We then amplify using this multiplex PCR with map page tagged primers. So that is done on a PCR. This is the ABO and RHD multiplex systems in two separate multiplex reactions, ABO and RHD, and everything else together.

In the later version of this we include all 10 RHD exons so the test is future proofed for any new RHD alleles that we certainly expect to find. So, we will be able to amplify every single RHD coding SNP using this assay.

(Slide)

Then the DNA is fragmented from the PCR reaction. It is then labeled either with biotin dUPT or Cy5 and then it is hybridized to the chip. And we

need very consistent hybridization and that is done on a ventana automated hybridization platform.

And then we read the array and that is a typical read out of our array. And if you have a very close look, you can see that you have variation in signal strength from your Cy3 signal to your Cy5 signals, and it is that variation in signal strength that allows you to score the genotype. So you will have a variety of different SNP scores, from wild type to the mutant and in the middle there are heterozygous.

And the software associated with the platform is able to detect which or what you have. Homozygous A, homozygous B or heterozygous.

(Slide)

Then we get a final output, which is read by software, and you will get positive scores for various SNPs and the software will then interpret the final phenotype of the donor that you get. So, the software is relatively intelligent and is able to predict the phenotype from the initial genotype.

(Slide)

Now, at present we are looking at C marking the kits, and this is the requirements in European legislation for C marking. We have to do 3,000 samples for ABO. We are not going to get it C marked for ABO. We are thinking of C marking it for RhD, but we certainly are C marking for RhC and KEL. So we have to do 1,000 samples.

At present we have completed those 1,000 samples, and that is the spread of sample types we must have. We have clinical samples, newborns, weak and variant RhDs and we have a 100 fully phenotyped samples by serology. So

those are ongoing.

(Slide)

That is the typical data from Willy's lab and accurate storing Weak D. So our serology predicts a weak D, and that is a genotype produced by the kit. That is the typical software that is produced by the kit, and we prove that we are concordant with the serology.

(Slide)

At present, and that was probably about three weeks ago, his results, we have some discrepancies. The major discrepancies are where the genomic DNA is insufficient quality. We get an intensity background readout. If that doesn't match what is required of the kits, then the kit will not score the genotype.

There are problems with the -- some issues with the software that we are working at as well. The genotype score is correct, but the phenotype predicted by the software was incorrect, and there are some general discrepancies between serology and genotyping, which we are going to assess by DNA sequencing. So, at present we are repeating those 154 samples to check what is actually going on.

(Slide)

So, if BloodChip gets used in Europe, we have talked about these patients before. Could it be that we have a comprehensively typed donor cohort, particularly for awaiting donors that come in?

I think Geoff Poole put it very well in the United Kingdom, who is national head of reagents provision in the United Kingdom. The Blood Service would like to be like Sanberry's. I guess you don't have Sanberry's here, but A&P. You have a variety of fully genotyped blood that is constantly available for these

rare donors that come through and require matched blood.

So, it could be a possible change in transfusion testing policy, and you could use it for those donors and recipients. So both donors and recipients would have to -- for the procedure to be highly effective you would need both patients and donors to be genotyped.

And, of course, we have already identified the multi-transfused patients that are a good target, and the detection of risky units in blood banks were already mentioned. But I think genotyping is going to be here for a long time, and it may well be that individuals would be genotyped shortly after birth, particularly for inborn errors of metabolism, and it would make perfect economic sense to genotype those people for blood group and HLA. So, I think genotyping is going to be very much part of our futures.

(Slide)

So, we are not -- we all right still a far way off I think for routing genotyping, but I don't think it is that far away, depending on the way you look at how things have developed in the last five years. We haven't devised all RH alleles, but we hope to discovery a few more in the project, and I am certain that those will be added to the chip, later versions of BloodChip.

Of course, getting an ABO call incorrect would be disaster, and I discussed with Marion Reid yesterday that ABO serology certainly I think is going to be here for some time yet.

(Slide)

So finally, I would like to thank the consortium members. Without their help or their input this project would have been absolutely impossibly. And

Willy is a member, a full paid up member of BloodGen, and is going to talk more about his experiences in Germany. So, many thanks for inviting me.

(Applause.)

MS. KOCHMAN: Now I would like to welcome Dr. Willy Flegel to give his talk on Blood Group Phenotyping in Germany.

Blood Group Genotyping in Germany

By Dr. Willy A. Flegel

DR. FLEGEL: Good morning. Thanks very much to the organizers for inviting me and for giving me the opportunity to talk about the experience of routine blood group genotyping n Germany.

(Slide)

As a start in Germany we have guidelines which mandate since 1996 that two anti-D monoclonals should be used that do not bind the D category six. These rules have been implemented in other European countries since we have the anti-D prophylaxis and the prenatal and postpartum setting. And for certain subgroups of our patients the other antigens, C/c, E/e are typed. That is certainly truthful for girls and women in child bearing age, people with multiple transfusions or other immnuhematologic problems.

Of course, the D antigen is most immunogenic because the D antigen -- actually, the --- is lacking in the D negative population, and that is the molecular basis of its clinical importance.

It is also the most important blood group system encoded by proteins, and on your right hand side you will see the number of alleges for the RHD gene only. It exceeds now 117, and this is the molecular basis behind those 49

antigens, as shown by a needle. It is 49 antigens for RHD and c/e, and this is the number for the RHD gene alleles only.

It is certainly going to expand quite significantly once these alleles on the patients are typed at the molecular level.

(Slide)

As a matter of fact we are doing this clinical application in a number of special cases, and I would like to show examples in the next slides. For example, the weak D donor and patient typing has been done since about the year 2000.

(Slide)

This slide shows our original results published in 1999 for the distribution of the molecular weak D types in Europeans, and as you probably know, the prevalent weak D types 1 to 4 comprise more than 90 percent of all weak D types in Europeans.

The least frequent weak D types are numerous. The number exceeds 54 by now, but the population frequency of those rare weak D types combined is less than about six percent in a European setting. This has often been confirmed in other European populations, in and outside of Europe. The details vary among European populations.

For example, in Portugese people the weak D type 2 has been observed most frequently. More frequently than the weak D type 1 that is the most prevalent in most other European populations.

(Slide)

If one looks to the clinical relevance of those weak D types, we found

that the prevalent weak D types are not likely to develop allo-anti-D. In fact, the prevalent weak D types 1 to 4.0/4.1 will not develop any allo-anti-D. At least we have not observed that since we started it since 1998. The only allo-anti-DSs were observed in weak D type 4.2, which is rare in European people, and weak D type 11 and 15, which are also rare.

The prevalent weak D types 1, 2, 3, 4.0, 4.1 and weak D type 5 were not observed with allo-anti-D or clinically relevant titers or other anti-D.

(Slide)

Therefore, the current clinical management of patients that I would recommend for weak D phenotypes is such that the prevalent weak D types should be transfused with a

D-positive blot, and as a consequence models should not receive anti-D prophylaxis if they carry one of the prevalent weak D phenotypes.

In difference to those patients and mothers who carry one of the rare weak D phenotypes are those weak D phenotypes that are known to be permissive for allo-anti-D production. Those patients or mothers should be transfused with D-negative blood and should receive the anti-D prophylaxis.

(Slide)

If this procedure would be applied, and we actually are recommending this and are applying it at our university hospital in Ulm, for example, or for patient samples sent in as reference material from all over Germany, this would save three to five percent of all of the negatives units. And these d-negative units not transfused to those patients who actually don't need d-negative units, these d-negative units for recipients who are really in need of them.

There is only one test required per patient or per mother and proper documentation assumed. There are

CE-labeled test kits available in Europe, and this service is offered in Germany since the year 2000.

(Slide)

Other clinical applications are applying to the prenatal diagnostics. There was a Germany consensus statement published in the year 2000 that blood group genotyping is standard care, and the puncture of the fetal cord should not be done if this would be pursued for blood group tying only. But it should be done by molecular techniques from the amniocentesis for example.

The typical diagnostic request that we get in Germany are in order of frequency for D, for the rhesus phenotypes, for the K antigen, Duffy kit, et cetera. It is possible to do the RHD genotype from maternal plasma, as will be presented by other speakers at this workshop. This is, however, at the moment not widely available in Germany.

(Slide)

So basically, there are two approaches for management of the -- of pregnancies. The RhIg prophylaxis during pregnancy maybe dropped and, as a matter of fact, should be dropped if the mother carries a certain weak D phenotype, as I presented before, and there is the possibility to type the fetus. And if the fetus is

D-negative, then of course no anti-D prophylaxis would be required.

(Slide)

And the health and cost benefits that are possible at the moment is

that about three to five percent of all anti-D shots could be saved if the mother is typed for weak D. There is only one test required in the mother's lifetime, and the test pays for itself. Therefore, the health benefit for the mother would come for free because you save a lot of anti-D shots that are also costing something.

The typing of the fetus from maternal plasma may save up to 40 percent of all prenatal anti-D shots. It won't save anything in the postnatal setting because this can be done by serology and is done by serology at the moment. There may be one or more tests required for pregnancy, and the test may perhaps pay for itself.

(Slide)

In regard to the weak D typing, as I said, we have CE-labeled the test kits. They are compatible with the current guidelines, as we are within the limits of the European and in particular the Germany guidelines when we apply such molecular tests, and this service is offered since the year 2001.

For the maternal plasma testing there are no test kits available. The current guidelines, at least in Germany, would need to be relaxed to permit this test. I would support this changing of the guidelines, but this change would also be required before we could implement the test system. Therefore, as far as I know, it is not currently offered as a service in Germany.

(Slide)

Another project that we started is blood donor testing for DEL, weak D and D+/D- chimera among so-called serologically D-negative donors.

(Slide)

It is by now very much established that the weak D, DEL and in

particular the chimera have the potential of immunizing D-negative recipients at least for secondary immunizations, but it has been shown for a number of instances that primary immunization is also possible.

And it should be remembered that not everybody who is carrying an RHD gene does necessarily the D antigen. For example, donors carrying the pseudo gene will not express the D antigen; however, a number of those donors carrying the RHD gene are able to express the D antigen in a weak form.

And it is not possible to detect those clinically relevant so-called D-negative donors at the serologic level. We would have to apply molecular techniques and these molecular techniques are established to improve the safety of RBZ units.

(Slide)

After a pilot study done in the year 2000 we established routine testing since January 2002 at blood service in southwestern Germany where we have more than 500,000 blood donations a year, and all first time donors are tested currently for the D antigen at the molecular level. We screen by now more than 30,000 units, and the screening is done with a PCR method for RHD intron 2 in pools of 20 donors.

If there is RHD allele encountered, then is allele is characterized either by PCR sequencing and if it is new, then we also test for the presence of the D antigen by allusion technique.

(Slide)

This is the result for our study. As you can see, most of the RHD gene positive donors are encountered among those donors with C or E antigen,

and those donors actually also are the donors who carry and express the D antigen in weak form. However, a D+/- chimera, because of the statistics, would be expected in the really D-negative population of c and e, and that is the situation now where we are testing all d-negative first-time donors.

(Slide)

These are the results of the frequency of alleles encountered. As you can see, this particular splice site mutation and this particular hybrid RHD allele is the most frequently encountered. Next is the pseudo gene and then there are a number of other alleles.

(Slide)

It is important to remember that the variation of the frequency of DEL phenotype is rather low in Europeans; therefore, it is possible to test with molecular techniques in pools. This is different in the African population where the number of RHD alleles among the negatives is much higher. And it is certainly different in East Asians where about 30 percent of all D-negative donors are carrying the RHD allele, and every third of this so-called D-negative donors are actually a DEL phenotype. The most prevalent one is the 409 allele.

(Slide)

Therefore, the RHD genotyping donors improves the RBC unit safety by removing donors from the D-negative donor pool and transferring this donor to the D-positive donor pool. This is compatible with current Germany and European guidelines. It has the advantage that it obviates the need for a very sensitive anti-D test at the serologic level, and therefore, a very strict quality assurance of the sensitivity is not required or would be not required anymore, which would infer

some cost savings.

The technology requires some adaptation to the allele distribution and in different populations. There have been protocols published for the East Asian population, as shown here on the slide, and we will publish later this year a different strategy also applicable to the East Asian population.

(Slide)

The next issue which we apply in a routine setting as anti-D is RHD zygosity testing in those parents when the mother carries an anti-D.

(Slide)

There are two technical approaches available. One is testing for the hybrid rhesus box as published in Blood in the year 2000, and the other approach is to test for the quantity of RHD alleles in a patient or in the father. And as you can see, the heterozygous carriers have a lower quantity or ratio of RHD to RHCE than those that are homozygous for the RHD gene.

But as we have shown in a publication earlier, this year in transfusion there are a number of alleles with very high ratios of RHDs and RHCE, and they are still heterozygous.

(Slide)

These are actually the technical problems that needs to be tackled if this technology is applied to the father's testing. This slide shows that the detection of a RHD heterozygous father -- if the hybrid rhesus box is present, in conjunction with a downstream rhesus box, then this determines that a father would be heterozygous.

However, there are a number of regulatory issues involved when it

comes to testing for the hybrid rhesus box or this dosage. In particular, since there are rhesus box variants known, primarily they are occurring in Africans. However, if you apply the zygosity test, then you run or may run into alleles that are actually hybrids, and these appear to occur primarily in Europeans.

Much more work needs to be done to test other populations. But as far as we did this work, we found it in the European population.

Further, resolution to this rhesus box variant is actually the dosage and there was allusion to this hybrid allele issue as the hybrid rhesus box testing. We agreed in Europe, and this strictly applies to the European and certainly the Germany setting, that we would recommend doing one test is better than no test.

Of course, the best would be to use both tests. When it comes to the hybrid rhesus box, we have CE-labeled test kits available, and we have offered this service since 2001. When we got to the one --- there is no CE-labeled test kit available at the moment, and we are not offering this as a service. Certainly not in Ulm.

(Slide)

In summary, we have quite a number of applications in routine applications since a couple of years, and we get a lot of samples sent in all of Germany to do this testing. And I am quite pleased that since the turn of the century we are able to offer molecular tests for blood groups as a routine service, and this came only a couple of years after it was predicted that this might be possible.

When I first predicted that in 1994, people laughed at me. But in the year 2000 we were at the point where we could already apply that to the benefit of

the patient, and it is very interesting to see that this is now taking a wider application.

And Neil trusts the issue; that at the moment it is possible to replace serology by molecular techniques. Legally and technically possible to replace a serologic test by molecular tests for a number of blood groups. We are probably not going to do that for the RHD gene, although it would be technically and legally possible.

It is not possible at the moment for ABO, but I am confident with the current development of techniques and the population of genetics behind it that in 10 years time it would be easily possible to replace ABO testing by molecular techniques. It doesn't mean that we will do at that time, but it would be technically and legally possible, as it is possible for RHD today. Thank you very much.

(Applause.)

MS. KOCHMAN: Now I would like to have Martina Prager come and give her presentation. Martina is from BAG in Germany.

Molecular Genetic Blood Group Typing by the use of PCR SSP Technique

By Martina Prager

MS. PRAGER: Good morning, ladies and gentlemen. First of all, I would like to thank Sheryl Kochman and the organizers for the invitation to this FDA workshop.

DNA based methods are useful to enhance immunohematology testing. Since the PCR SSP method is well known and utilized for many different applications in many labs, we decided to develop test systems which enabled to

examine weak, unexpected or unclear serologic findings by the use of this easy-to-handle technique.

(Slide)

The next slide shows an overview about the following presentation. After a short introduction of our company, test principles and procedure of the PCR SSP technique will be explained, followed by aspects of development, validation and studies using CE marked test kits.

I will finalize the presentation highlighting the intended use and the application of PCR SSP kits in immunohematology.

(Slide)

BAG is located in a nice and scenic little village in the middle of Germany, and Lich is also known for a very famous beer. Of course, only in Germany, not in the U.S.

(Slide)

We are in the market since more than 50 years, and we develop, produce and sell diagnostics for transfusion medicine, transplantation medicine and for infectious diseases. We have a quality management and assurance in terms of DIN EN ISO 9001, and our in vitro diagnostics are conformed with directive of the European parliament on in vitro diagnostics. That means all of our in vitro diagnostic products are CE-marked.

(Slide)

The next slide shows a short sketch of the PCR SSP test principle. The method is based on the effect that primer extension, and therefore, successful PCR relies on an exact match at the three primes --- forward and --- primers.

Therefore, only if the primers entirely match the target sequence amplification is obtained, which is subsequently visualized by --- gel electrophoresis.

(Slide)

The next slide shows the list of contents of our DNA SSP kits. PCR plates or strips with pre-aliquoted, dried and colored reaction mixes containing allele specific primers, internal control primers specific for the human growth hormone gene and nucleotides.

PCR buffer, PCR strip caps, worksheets and evaluation diagrams and, last but not least, instructions for use.

(Slide)

The next slide here you can see the steps of test procedure, beginning with the DNA extraction using well-known techniques, such as --- or salting alt procedure. The next step is the preparation of Mastermix, followed by the PCR reaction itself, followed by gel electrophoresis and the evaluation of results. So, results can be obtained within approximately three hours.

(Slide)

This table explains in detail the preparation of the Mastermix, depending on the number of reaction mixes. DNA solution, water, PCR buffer and --- polymerase have to be pipetted into a tube and mixed well. Afterwards 10 micro liters of the Mastermix have to be added to each well of the PCR plates or strips respectively.

(Slide)

Here we can see an example for the evaluation of results, showing a worksheet for Rh genotyping, and on the front page one can see the specificities,

the PCR product sizes and the reaction patterns. Positive reactions, that means bands in the gel, are indicated by "+" signs in colored boxes, whereas negative reactions, that means absence of specific amplicons, are indicated by a minus in a wide box.

And on the back page here you can see a short instruction for us and the table for the preparation of Mastermix. And here you can see some for gel pictures.

(Slide)

In the following six slides DEL pictures and reaction patterns of different blood group systems are explained. A selection of scientific publications on which the development of our test kits mainly is based on is added to each test system. Of course, an extended list of references one can find in our product inserts.

Common and rare alleles of the Caucasian population, as well as some antigens specific for African or Asian populations can be identified. But more detailed information regarding the specificities you can follow up later in your handouts.

The principal of evaluation of results is identical in all of our SSP kits, which means that the upper bands indicates the internal PCR control whereas the lower bands show the reaction of the specific alleles.

We start with genotyping of the ABO blood groups, the determination of the main alleles. A1, A2, BO1 and A2 can be performed using BAGene ABO type kit, but we also offer an additional kit. It is called ABO type variant for typing some rare A and B subgroups, which you can see in this slide.

(Slide)

This is an example for molecular genetic determination of standard RHD and RHCE alleles, as well as the typing of a few RHD variants, which is shown in this slide, such as the pseudo gene or DEL types and the CDS specific in the African population.

(Slide)

The next slide shows the partial D type kit which allows the molecular genetic determination of D categories, as well as the RHD/CE hybrids, and furthermore, RHD variants, shown over here.

(Slide)

The most common as well as rare weak D's can be determined using the weak D type kits.

(Slide)

Determining the RHD Zygosity is an important tool for prenatal diagnostic purposes. The positive detection of the downstream rhesus box stands for the presence of the RHD gene, whereas the hybrid box detects the deletion of the RHD gene.

(Slide)

A clear identification of K, k, Kidd A and B, Duffy a and b, as well as the GATA mutation and the Duffy b weak can be achieved using the KKD-type kit.

(Slide)

The next slide shows development issues. First of all, beginning with the new product idea we have, of course, to create the design to see and all of the required documents for the -- oh, there is something wrong with the CE. It means

CE-marking and in vitro diagnostics for blood group typing on a molecular genetic basis, such as the kit designs, specifications, milestones, SOPs, product inserts, labeling, packaging and et cetera.

(Slide)

The primers were designed according to mutation databases mentioned in this slide. Published primers were first checked for sequence similarities using the BLAST database and subsequently adjusted for uniform melting temperature. After pilot experiments alternative primers were tested if necessary.

The selection of clinically relevant alleles was performed according to extensive discussions among scientists from Germany, Austria and Switzerland, joined in a working group belonging to the DGTI, the Germany Society for Transfusion Medicine and Immunohematology.

The final step is the verification of design using prety samples.

(Slide)

The next slide shows the validation procedure. First of all, we have to produce a prototype, and with this prototype we have to perform a risk analysis, followed by stability testing and then the most important issue is the proficiency testing using approximately 1,000 in-house and external samples.

The external samples we gratefully receive from different universities. From Professor Flegel in Ulm, Professor Selsum in Hanover, from Professor Legler in Gottingen, in Germany, and from Lund and Sweden. And after the registration procedure the new product is ready for launch.

(Slide)

External studies are presented in this slide. The first publication is a comparison study of serology and SSP technique of 950 blood donor samples performed by the University of Mainz in Germany and presented last week at the DGGI Congress. The next study of the university Gottingen in Germany deals with ABO and RH genotyping of blood donor samples from South Africa and was presented at the DGGI Congress last June.

A summary of further studies in other countries than Germany will be presented in the near future. For instance, there are studies in Korea, Italy and the Czech Republic.

(Slide)

Now I will figure out the intended use of SSP kits. The molecular determination of blood group antigens using SSP kits has to be performed after serology, and we offer these assays as a supplementary technique to investigate weak or discrepant serologic findings. But the current assays cannot replace serology.

In case of discrepant or unclear genotyping results, transfusion guidelines have to be followed in accordance with serologic typings and final clarification by sequencing analysis is recommended.

(Slide)

Now we come to the different applications for the SSP kits. Genotype multi transfused recipients, genotype patients after ABO-incompatible bone marrow transplantation and determine RHD zygosity of partners of alloimmunised D-negative women before pregnancies. Genotype RHD negative donors was, for example, C or E, in order to exclude the presence of RHD gene, and that is

preventing anti-D alloimmunization of recipients caused by hidden RHD variants in RBC units.

Identify genotype in case of weakly expressed RHD. For instance, DEL types in donors. Confirm weak D genotypes in recipients in order to avoid the donation of RHD negative blood units. And it is suitable for quality control of serological methods, and it is suitable as well for external quality assurance trials.

(Slide)

Now, on this slide one can see an investigation strategy for the ABO blood group typing. Beginning with the serological determination, one has to follow by molecular or genetic typing because a clear ABO blood group typing cannot be achieved, for instance, with samples of polytransfused recipients. And weak expression of A and B antigens either associated with normal or with unexpected reverse typings also hamper the evaluation of serologic results.

So, the ABO type variant kit allows to reserve most of unclear serologic findings and, of course, you can use the kit also even when you have unambiguous results for the confirmation of your serological findings.

(Slide)

The next slide shows an investigation strategy for the rhesus typing. Unclear RH phenotypes can be investigated selecting SSP kits depending on specific purposes. Here we have an approach on how to proceed in case of a questionable partial D pretype serology.

Quite often a weak D instead of a partial D is hidden behind. That is why we recommend to test for the weak D's first, and in case that weak D is excluded, proceed using the partial D-type kits. An additional kit is available to

examine D-negative samples. For instance, with a C or a E. The RH kit enables to detect D-negative RHD alleles, such as DEL types or the or the RHD pseudo gene or CDES. And, of course, RHCE antigens can also be cross checked using this SSP kit.

(Slide)

Coming to the conclusion, the SSP technique is helpful to resolve most of the problems caused by discrepant or doubtful serological results, and it is an easy to handle technique. Questionable cases in donor, recipient and patient typing can be examined with acceptable cost. It is not suitable for high throughput, and it is not suitable to replace serology. Thank you very much for your attention.

(Applause.)

Panel Discussion

MS. KOCHMAN: We are going to move to the first question and answer session. So, if the speakers would come on up.

(Pause.)

MS. KOCHMAN: I am going to start off the questions myself. Dr. Flegel, you indicated that for your testing of women one test per lifetime would be sufficient. In my mind that presume that you will always be able to tell when that woman presents again, that she is indeed the same woman that you typed in the past. How is it that you are able to do that in Germany?

DR. FLEGEL: Well, it assumes that there is a proper documentation to be done and that we have proper identification of the individual, but this should generally be possible if a proper medical documentation and file is established for a patient, as it is done for a lot of other medical diagnoses in Germany.

MS. KOCHMAN: I think that in the United States we are aware of a number of cases where patients -- where multiple patients with the same or very similar name may be in the hospital at the same time. I don't know that we have found the answer to necessarily dealing with that at this point. Do you have any biometrics available or anything like that? Or are you simply talking about very good documentation?

DR. FLEGEL: Well, if a molecular test is done with clinical consequences as detailed, then we would issue a patient card, an identification card with this molecular test documentation. And if the patients come in with that card and on top of it they can identify, by other means. as the person having this diagnosis, then I think it is very unlikely that there is a mix up in regards to this identification issue.

Let me add that at the moment there is no final decision how often the molecular test would need to be repeated to establish such an identification card. The moment we do it once at the molecular level it might be necessary to do that in the future twice. Perhaps from independent samples or twice with different molecular tests.

But there are no rules in the moment in Germany and, as far as I know in Europe, that more than one molecular test needs to be done, but that rule may change in the future as it is the current practice, for example, for the serology testing where we need to do the test with two independent monoclonal antibodies.

DR. GARRATTY: George Garratty from Pomona, California. Neil, considering the politics in Europe -- and I relate more to the UK, and I have gotten in several arguments at dinner tables in the UK about genetically modified food

stocks and crops and they seem quite -- there seems to be a lot more reaction against them over there than here.

And I saw you just released -- I saw a press release just before I came here about that. Do you envisage or have there been any concerns from the public about you playing around with that DNA?

DR. AVENT: Well, of course for the moment there is no disease associations that we know of. I know you have looked into that research extensively, George. But there are no harden fast defined disease associations with blood group alleles. So I think the information that we produce on BloodChip would be of only a benefit to blood donors and to patients themselves.

So, the information, yes. It potentially could be used for other purposes, but at the moment those associations aren't there. And I take your point. In the UK we have got something called the Human Tissue Act, which has just come into force the 1st of September, and there is an issue of DNA theft.

If we test an individual's DNA without their knowledge, then we could be banged up in prison. So it is very much a very hot political issues back in the United Kingdom, and I think that will probably push forward into Europe as things tend to go that way.

DR. GARRATTY: Well, it does. In other words, you haven't had anything yet, but you might get some stirring of the matter later.

DR. AVENT: Yes.

DR. MENY: This is Geralyn Meny, from the Red Cross in Philadelphia. Have you actually informed any donors yet of testing results? If you have had the serology results and now the molecular testing has changed a result

and now you have to tell them something different and you have to clarify the result or tell them they can't be a donor and now -- have you provided any information and now given them the result and counsel them in a way that you are recommending now in the future as a patient they should receive a different type of blood? Or how are you doing that?

DR. AVENT: Well, Willy was actually in charge of the ethics of the whole consortium. But certainly, the United Kingdom is completely anonymous. So we do not know who the donor is. So that information can never get back to the donor. Willy?

DR. FLEGEL: Within the project you just addressed, when it comes to patient or donor testing in a regular blood donor or blood service situation then we often go back to the patient and to the donor, sometimes with the information that the information has changed relative to the knowledge a couple of years ago, and the patients, as well as the donors, are happy.

That is what I can tell you from 12 years, 14 years experience in doing this at the serologic and molecular level. For example, the D category six thing with the monoclonal antibody we frequently have changes or weak D types are now differently categorized than a couple of years ago.

And if donors or patients are addressed with this issue, they are happy to hear that news. They may have some questions, but these questions can be answered and they are quite happy to get the latest technology applied to their health benefit. I think that applying molecular techniques at the immunohematology level actually promotes the issue of genetic testing in the human population.

There are not that many ethical or legal issues involved, and those ethics and legal issues can be dealt with much better for any of the genetic testing. Like, for example, a tumor oncogen testing. It is much complicated than doing it at the immunohematology level, and therefore, we should use genetic testing for immunohematology to make it easier for the public and the rightful concerns of the public to learn how genetic testing is applied to the benefit of the patient and the donor.

DR. RIOS: Maria Rios, CBER/FDA. One question regarding you performing genotyping for blood group as a service, but as a research activity. Is that correct?

DR. AVENT: Personally, it is completely research. But other than the consortium, we don't provide that as a routine service for their donors.

DR. RIOS: Do you need an internal review board to approve evaluation of using molecular testing in providing results for clinical use? How does that work in Europe?

DR. AVENT: It works by C marking. Once the kit is actually C-marked, then it is used for in-vitro diagnostic use. That is part of the legislature which you had mentioned in the E-Blood Directory, the in vitro diagnostics directory, which was mentioned earlier this morning. So, that is the situation in the European Union.

DR. MOULDS: Joann Moulds, LifeShare Blood Centers, Shreveport, Louisiana. My question is for Dr. Flegel. You quoted one in 1,000 as the incidents of DEL, but I think the data that is missing here is how often are those antigenic? Have you or are you aware of anybody that has gone back and looked at those RH

negative recipients to see if they indeed do make anti-D?

And the second part of that question is if that has been done, how often is it a woman of child bearing age, which is really the ones that we are most concerned about?

DR. FLEGEL: The frequency of the RHD gene was one in 500. The frequency of DEL, of weak expressed D antigen, was one in 1,000. Of course, we are doing first-time donor testing. Therefore, it is not possible to do any look backs because there is no previous donations.

In our pilot studies we did look backs and in those limited number of look backs there was no immunization by the DEL units involved, but we found frequent -- and I discussed that a couple of times at various conferences where repeated immunizations with a D+/- chimera donor detected amongst our D-negative repeat donors in the year 2000.

So, I don't have definite numbers. Obviously this needs to be established. In the literature you will find one documented case of a likely primary immunization in a pregnant woman as a matter of fact. Of course, the numbers are limited, but even with the current limited knowledge we have already a documentation of a likely primary immunization in pregnancy.

DR. WESTHOFF: Connie Westhoff, from the American Red Cross. Neil, I wondered if you would comment on what seems to the novice here a rather high 152 samples that were discordant or discrepant. Do you anticipate those are new alleles or maybe procedural changes required?

DR. AVENT: Yes. They are a little bit more detailed in the handouts I gave. Those 154 really are samples that need repeating because they didn't get

the intensity background scores. There was something wrong with the control DNAs in that kit. So the whole run was stopped, although the genotype scores were highly accurate.

There are some software needs to learn. The software was producing an incorrect phenotype although the genotype score was correct. So, it looks actually bad, it is not that bad at all. In fact, the genotype is 100 percent accurate for those scores that you can officially pool.

Some of them there are going to be serology errors. So there are errors in the serology; in transcribing the serology data into the genotyping software. We found some errors in that as well. So, there are a whole host of errors that we are finding out as we go through. But those 154 samples will be retested to see that the genotypes are correct as we predict.

DR. STRONECK: Dave Stroneck, at Bethesda. From a transfusion point of view sickle cell patients, the RHCE phenotypes, and we seem to be seeing a relatively high incidence of antibodies to variants C and E antigens. It doesn't look to me like your kits and their arrays are geared towards picking up RHCE variants, at least not to the degree that would be helpful with assessing patients with sickle cell disease. Has anybody tried a direct sequencing approach for the C and E genes anyway to address that issue?

DR. AVENT: I think it is only partially understood, the C and E variation. I think we have a few alleles for C and E on BloodChip, and certainly I think it is an area that could be investigated and additions could be made to subsequent versions of the testing platforms that we have available.

As we understand the model basis of those antigens, and particularly

in sickle cell patients in African populations, those alleles are really just coming to the surface. I think in the last two or three years some of those have actually been described. Perhaps Willy has some more information.

DR. FLEGEL: No. Sorry. I do not.

DR. STRONECK: Okay. I guess a follow up question. At least for a diverse population like in the U.S. it is really important to be able to pick up the polymorphisms found in the various ethnic groups. Are you making any special efforts in your European studies to weight your people you look at towards non -- people of non-Caucasians?

DR. AVENT: Well, certainly the population groups that have been tested in the United Kingdom and the Netherlands are very diverse, but there was no specific weighting toward one particular population group. We randomly selected. It was random; 160, 170 donors from each center.

But I totally take your point. It is something that we need to address, is how are these alleles -- how is the performance of these tests going to behave in very diverse ethnic groups? And I think certainly we are looking at getting funding from the European Union, and that is one of the topics that we want to address; is to look at these various population groups and see the frequency of novel new alleles that may be out there in the population. And the frequency of new ones as well.

You just don't really know, at the end of the day, what those frequencies are. There are still some questions to be addressed.

MS. PATTISON: Paula Pattison, Ortho-Clinical Diagnostics. This is a question maybe for the panel. I'm not sure who to direct it to, but perhaps Dr.

Flegel. And it may be a question of the mechanism of the immune response. I don't know.

But I continue to be perplexed and continue to question if we are able to demonstrate the presence of a gene through any of the techniques we have available today, but we are in, addition to that, able to suggest that the antigen, whether it be the protein or carbohydrate structure, is not expressed on the cellular component, do we know if the recipient or the person who is exposed to the genetic difference will respond immunologically?

DR. FLEGEL: We don't know the answer, and that is a very interesting point to do further studies in the future. It is a very interesting point. We assume that a donor, negative in the allusion technique, is not capable of causing an anti-D response. This may not be true.

Of course, we introduce another serologic cutoff because we want to get rid of serology in that respect. So perhaps we are wrong with assuming that DEL only is capable of producing anti-D. Maybe the other seemingly really sort of D-negative alleles are also capable of inducing some sort of adverse response. We don't know at the moment.

DR. AVENT: I would like to answer that. Certainly Stan O'Baniack's work from Aberdeen has shown that HLA type if pretty important. Other factors, like subsequent gene polymorphisms, may preclude a certain sub-population of the D negative population to make much stronger anti-D or much stronger immune responses.

So I think my point in my talk, HLA Type is always going to be useful information. Maybe that approach of looking at someone's HLA genotype with their

blood group genotype in the space of time would give us a good inkling of how that person's immune response is going to react when it is challenged.

Some people may respond very, very well to a particular transfusion or very badly. It depends which way you look at it. Some may not respond at all. Maybe the traditional term of non-responder may be true. So I think HLA is certainly something that needs to be considered in the future, which is something blood bankers, I think, have ever really considered in transfusion.

MS. PATTISON: I have one more question for Willy. I have heard you mention several times a population-based approach to some of these testings, as in Asia and some regions, and I wonder how comfortable anyone would really be with that in the long-run when you consider the mobility of populations and add mixture and, you know, different population bases that we have no idea of their true origins. Is that really a selling point in your --

DR. FLEGEL: Well, we do as good as we can. We look into -- certainly into different populations, like the Europeans, like the East Asians and Africans, but it is also necessary to look at mixed populations obviously. And you here in the U.S. have the best possibility to approach that issue once you start doing that in greater depth.

I absolutely agree with your point. There is relevance in looking at the less mixed populations, like Europeans, East Asians and African populations. Particularly the latter one hasn't been explored in much depth. This 170 alleles basically represents the Euro Asian allele pool, plus the more obvious African alleles, but there are many, many more African alleles out there.

The total number may well exceed 1,000. And so, we just have the

tip of the iceberg. Perhaps the more important ones, but we actually don't know. And the future studies will reveal the whole complexity and further improvements in those techniques are required. In particular when it comes to sickle cell patients, a treatment which is basically an African allele issue.

MS. KOCHMAN: Martina, I had a question for you. You indicated that currently your tests are not to be used without having done serologic studies first. Do you envision that that intended use will change over time as you gain more information?

MS. PRAGER: I don't think so because, as you know, the SSP technique is not comparable with a sequencing analysis. And so, we have, of course, some imitations in some specific SSP kits. We never will overcome this so that we can replace serology by the use of this technique, and we don't recommend it.

We recommend to use the SSP test kits for specific questions, as I mentioned in my presentation, but we don't intend to replace serology by these test kits.

MS. KOCHMAN: And, Christine or possibly anybody else who has been to the ISBT/ICHS meetings, you indicated that it seemed everyone who participated had their own favorite method, which doesn't help us get a standardized test available.

So, this is obviously a projection of do you think, as people gain experience, they will focus more towards fewer methods? Will what is available commercially on the market drive what people are using? Or do you have any sense on where that is going?

MS. LOMAS-FRANCIS: My guess is that all of those things are going to influence the tests that are being used. I don't know what everyone else's opinion is, but if kits are available that are validated for certain use, then that will be the method that is being used.

But other times people are going to use a test that they find gives the best result for a particular SNP. Not all SNPs are equal in the way that they are tested for. People are going to use tests that they controls for or they are going to use real-time PCR because they have the piece of equipment already in-house.

I think it is going to be a while before this all shakes out. But there typically are going to be different techniques being confined for the different alleles. I don't know what the other members of the workshop would say to that.

MS. KOCHMAN: Thank you.

MS. FIGUEROA: Dolores Figueroa, Blood Systems. Christine, this question probably is for you. You talked about a lot of clerical errors that were in those samples that were evaluated by the different laboratories. But the other type of errors, are they technical or most problems with the DNA extraction or due to the methodology used for the testing?

MS. LOMAS-FRANCIS: I think it is not possible to say if they were DNA extraction or something like that. The organizers collated those results as people entered them into the worksheets. Some of them, as I mentioned, were due to the fact that, for example, the patient was reported as being IHD positive, whereas the patient could be expected to be

D-negative because there was no testing for the pseudo gene. Similarly for Duffy. There was no testing for the silencing mutation.

Then there were hours of transcription where in one part of the response the correct test result had been entered, correct as far as the consensus was concerned. But then another section which was concerning the reporting out. It was interpreted incorrectly.

So, the errors were really of many different types, but once it could be overcome, then, as people become more experienced in the testing that they do, I don't think they were related that much to the technique, but more not application of additional techniques or not of interpreting a wide enough picture.

DR. MOULDS: Joann Moulds, Shreveport. I would like to comment on that further, having participated in the workshop. For those of you who are not aware, there was no standard terminology to report it out. Everybody reported their own terms, their own way of reporting it and then kind of a consensus was taken.

So, some of those "errors" may have been technically correct, but they just didn't report it out the same way everybody else reported it out. So, as Christine says, there are a lot of different things that come into play here that really or may not have been considered as real errors.

DR. RIOS: Maria Rios, CBER. The European consortium, the goal of the consortium, is to validate and standardize molecular genotyping approaches for large-scale blood group diagnoses. And according to your slide, it said to prove that molecular genotype is superior to the currently applied serology testing.

For all of you, do you envision that you really will replace completely serology by genotyping, and how would you predict that a change in a gene sequence will always have the impact in a phenotype expression? How can you discuss that?

DR. AVENT: I think in the long-term it will replace serology. I personally don't have any doubt of that, but I'm sure there are a lot of people in the audience that will. I think as long as we catalog all of the genetic variation that causes blood group phenotypes then we will have a situation where we can arrive at a fully comprehensive genotyping platform.

That is what need to be done. So, what has kept us in jobs for 10 or 15 years, those of us who have been genotyping, we know that we have to do that. In terms of ABO serology, of course, at the moment that is extremely accurate and extremely cheap, and clinically, getting one of those wrong, the genotyping, if genotyping were to replace serology, is something that cannot be done.

Of course, for other blood groups serology is vastly out performed by genotyping, and our feel is that comprehensive genotyping -- that you have a platform that you can genotype a large cohort of donors and provide information for blood banks that have 2,000 to 3,000, 4,000 or 5,000 of their donors comprehensively genotyped. I think that is going to be a huge benefit.

DR. RIOS: Are you planning on trying to genotype the patient so that you can provide better patient care?

DR. AVENT: Well, that is an offer I guess. If you have a patient that is multi transfused, you have to match the donor cohort with the patient. The patient has to be tested in the same manner, otherwise the system is not going to work. So, in the end it would be hoped that those vulnerable patients would be fully genotyped as well.

MS. : Gayle from Seattle. At what level do you match them? You can't match them 100 percent especially if you are throwing in HLA. I

mean, you would be finding a family member by then.

DR. AVENT: Well, the capacity on the chips. We are only using a small percentage, and I guess that is true of all of the technical platforms that is available. We are only using a small percentage of the capacity on those chips. And certainly, if you look at other high throughput genotyping testing platforms, you can detect millions of alleles using the Alfie Metrix (sic) platform.

So I think it is more the issue of how willing are we to sequence blood group defining genes from large numbers of patients, which would have to be done to catalog those new polymorphisms and genetic variations. I think that is going to be the major limiting factor. I don't think the capacity of the systems is going to be an issue.

It could be that certainly alleles are clinically negligible, and to a certain extent the alleles that we have selected on BloodChip have been driven by that clinical significance. So I think the interpretation of data, that you could get far too much data, and really, you only need to unravel which is of clinical significance at the end of the day.

MS. LOMAS-FRANCIS: I was going to follow that. As Neil alluded to, we need to figure out what is going to be of clinical significance because you will have so much information. We have already talked to some people and they say, I don't want to know all of this.

Well, we need to know what is going to be helpful, but we still have another major factor to overcome and that is simple statistics. You know, we can be screening all of our patients and we can maybe find out -- I was going to say --- negative, but we don't know the molecular basis.

But we can find that they lack some -- are likely to lack an antigen to a high prevalence antibody. Well, that is not going to help us that much, in the first instance, to match their genotype to a donor because we are dealing with some aspects that the donors are still going to be very rare.

We are now improving our chances if we can do high throughput testing. We are going to test more donors. We may find more donors. But, you know, whatever information we find out, we have to decide what we really need to address at that point of transfusion, because we may not be able to find a donor that matches because of the statistical commons of some of these.

MS. KOCHMAN: Well, if there are no more questions, I think we can move to our break out in the lounge area. And if everyone could, come back here by 11:00. Thank you.

(Whereupon, a brief recess was taken.)

MS. KOCHMAN: I have one announcement before we get back to the meeting itself. I forgot to include Dr. Don Siegel's name on the list of speakers in my acknowledgment. So, I want to make sure that he gets recognition also. He brought his handouts with him. They are down here on the front table so that you can have easy access to them and take that with you and add it to your packet.

I am really honored to be able to introduce next Dr. Marion Reid. As many of you I am sure know, Marion and Christine Lomas-Francis coauthored the Blood Group Antigens Fact Book, and she is also a coauthor with Greg Denomme and Maria Rios on Molecular Protocols in Transfusion Medicine.

She has a long list of awards that she has received, the most recently and probably most prestigious one being the 2006 International Women in

Transfusion Award, and I'm thrilled to death to have her here and grateful for all the work she has done with me on this. And so, I will turn it over to Marion.

Overview of Molecular Methods in Immunohematology

By Dr. Marion E. Reid

DR. REID: Good morning. Looking out onto the audience, it looks like I'm speaking to a party of my extended family. I would like to thank the organizers for inviting me to this meeting, and particularly to Sheryl for embracing the idea and organizing it. It is a tremendous amount of work. She has done by far the lion's share. So, thank you, Sheryl.

(Slide)

I am going to give you an overview and talk about these items, and a lot of it will be repetitious and what you will hear here, but I am hoping it will give a framework to discussions later on today and tomorrow.

First, I would mention the limitations of hemagglutination, that there is a need for a larger inventory of antigen negative red cell components, that there is value in microarray technology, there is value in testing by DNA for fetuses and patients and donors, there are limitations to DNA testing and then I will talk about some other considerations or points for discussion.

(Slide)

So, why in the heck would we want to change from hemagglutination, which is just fantastic? I love it. It is my favorite technique. It is positive or it is negative. That is it. You don't have to worry about anything else.

(Slide)

However, it does have limitations. It only gives an indirect indication

of a fetus at risk for hemolytic disease of the newborn, it is difficult to phenotype a recently multiply transfused patient, it can be difficult to type red cells that are coated with IgG, and a relatively small number of donors can be tested for a relatively small number of antigens, which has limited the antigen negative imageries that we all have and have already talked about.

And you cannot reliably determine zygosity and it is a subjective test.

(Slide)

One of the reasons I am interested in it, and I think a lot of people, is to help the sickle cell patients. There are obviously others, but the sickle cell patients. The STOP program, which was a program to give transfusions to prevent strokes in sickle cell patients. The first STOP program was terminated 18 months early because it was obvious that continuous transfusion did prevent the strokes. The trial was so successful that it was obvious of its value.

The second STOP program was a six-year trial, and it was aborted after two years because, again, transfusions just were -- it was unethical to withhold transfusions. Of 41 patients that were selected to discontinue transfusions, 14 reverted to high risk transcranial Doppler and intercranial images, and two suffered strokes, whereas 38 patients who continued to receive transfusions, none had strokes and none reverted to a higher risk status.

So, it was stated that withholding blood transfusions, because of the risks, those decisions have to be made on a case-by-case basis. You can't just say transfuse this patient. And the case-by-case basis really comes down to alloimmunization and the problems that that has created for us.

(Slide)

So, if we are going to transfuse these patients effectively, we need to find better ways to reduce the risk of their transfusion reactions, the hyper-hemolysis system and alloimmunization. So I think for the first time in the history of blood transfusion the microarray technology has given us a tool whereby we can accomplish this.

(Slide)

So, hemagglutination. Not necessarily limitations, but the -- it is a very labor-intensive technique, not only in the testing, but also in data entry. This is for the minor antigens. The source material is expensive and is diminishing in its availability. The cost of commercial reagents that are FDA approved is escalating at an alarming rate. At least for me.

And it depends on whether you want to use the FDA approved for screening and for confirmation or whether you use a home brewed for screening and then confirm. But if you use it for the screening, it can cost you a tremendous amount of money to get a reasonable antigen negative inventory.

And then many antibodies that are not FDA approved are characterized often partially by the user. Certainly some people will characterize them to a greater or better extent, but they may not contain only the antibody that you think you have. So, there are problems with that approach.

(Slide)

And some antibodies are in limited volume, weakly reactive or just not available. And because the genes of the 28 or 29 blood group systems are being sequenced, the molecular basis of the vast majority of antigens and many of the phenotypes have been determined so we can use DNA assays to address

problems that we couldn't address before, and the reagents do not require any special or human source material.

You can buy the reagents very easily.

(Slide)

So, when can we use molecular techniques in transfusion medicine? We can use it for antigen typing to identify the fetus at risk for HDN. We can use it in patients, those that have been recently transfused and those that have a positive direct antiglobulin test.

We can use it to screen for antigen negative donors, and we can use it to type reagent red cells, both screening and identified.

We can determine the zygosity, particularly RHD. We can resolve discrepancies, we can identify the molecular basis of unusual serological phenotypes -- I think that is my favorite -- we can develop one-step automated objective antibody detection identification methods, we can use transfected cells for immunogens, we convert IgG monoclonals to IgM directed glutinens, we can even determine the origin of engrafted leucocytes in stem cell recipients or determine the origin of lymphocytes in patients with GVH disease.

(Slide)

Now, for the fetus at risk for HDN it can predict the antigen type of the fetus. That is the power of it. The source of DNA usually today is amniocytes, but become more popular is maternal plasma. It amazes me that you can get a sufficient amount of fetal DNA from maternal plasma, and it is particularly of value if the mother is RH negative due to a deleted D gene. It is relatively easy to find the D-positive fetus.

I would like to make a recommendation, after being caught a couple of times with other people doing this testing, that whenever you do a DNA testing for any type, that you always do RHD and this preempts requests for D-negative should there need to be any in in uterine transfusions. It is much easier to provide D+c negative blood than D-c, negative blood.

The potential pitfalls include contamination by internal by maternal DNA and false negative results because of the assays we might choose to determine any particular gene.

(Slide)

For DNA testing in the patient we can do it for those who have been recently transfused, as I have already said, and those that have a -- red cells that have a positive DAT. We can use it to distinguish allo from autoantibodies. We can detect weakly expressed antigens, such as the Fyb and the Fyx phenotype, and if the patient is of that type, they are unlikely to make antibodies if they are transfused with antigen positive blood.

And we can use it to identify the molecular basis for unusual serological results, especially RH variants.

(Slide)

We used to guess the phenotype of a patient if they were being transfused based on the strength of the hemagglutination, the number of red cell components that had been transfused, how long it had been since they were transfused, the estimated blood volume of the patient and the prevalence of the antigen. We were wrong more often than we were right.

We did this study some years ago and showed that the DNA from

urine sediment, buccal smear or peripheral blood gave the same results, and it was consistent with the pre-transfusion hemagglutination test, and obviously donor white cells -- sorry. The donor white cells that may have been present did not interfere with the PCR assays in the way we perform them. Now, if you want to look for chimerism and do very sensitive tests, you can find chimerism. But in the way we do the test we have never had a problem.

(Slide)

If a patient has a positive direct antiglobulin test, it is useful when direct antigglutinatng antibodies or murine monoclonal antibodies are not available, when the antisera or weakly reactive, when antigen is sensitive to the IgG removal treatment and to distinguish allo from autoantibodies.

(Slide)

And with the microarray ability to test a lot of donors, the power would be in testing for antigen negative donor inventory. This is particularly useful for antibodies that are weakly reactive or not available. Anti Doa and Dob are classic examples. Anti Jsa is another. Anti Jsb. We have a monoclonal antibody. So anti V and VS and I put anti S/s in tongue in cheek; however, there is one donor for anti S, and a couple of years ago that donor got sick, and it is very difficult to get reagents.

They are often on back order. So sometimes if we can do the molecular testing, screen this way and then confirm with our precious reagents, maybe it will make common sense. And mass screening can be done to increase the antigen negative inventory and to find donors whose red cells lack a high prevalence antigen, and you can do that simultaneously.

We can test reagent red cells, and I will go into that in a bit more detail, resolve discrepancies and detect genes in coding weak antigens.

(Slide)

So, to test donors for commercial reagent cells, we have done several years ago on an in-house panel so we can predict what the antigen type of those red cells would be when we don't have the reagents. We can confirm zygosity, and this is particularly important with S, D, Fya/b and Jka/b. We may think we have the homozygote cell, where it is actually a hemizygote. One gene expresses the antigen, the other gene is silenced.

And we can use it to define, which we found very useful, the specific Knops antigens negative for a specific antigen versus having a lot copy number of the protein that carries the antigens. Is this desirable, is it recommended and should it be mandated?

(Slide)

In testing for antigen negative donors, like all donors antigen negative donors must be tested for ABO and RH. It is a no-brainer. But due to the complexity of the ABO and RH blood group genes, as you have already heard this morning, DNA analysis is not the method of choice for routine ABO and D type determination.

The screening of donors for minor antigens is extremely valuable, and as I have said before, we can conserve the precious reagents to confirm DNA testing.

(Slide)

So, why do I say we shouldn't do ABO and D by DNA testing? The

naturally occurring anti A and anti B in the plasma of most people who lack the corresponding antigens provides a built-in control. It is a nice check. So when we perform the ABO typing, we are pretty confident with the hemagglutination of what type of it is.

We have potent well standardized monoclonal reagents. So they are available in volume. It is a relatively simple and rapid test. The systems are in place to test and record relatively efficiently the ABO and D type of a donor, and there are few antigens, but many alleles. As you have already heard, in the ABO system there are two antigens for allele, but well over 100 alleles.

As you have heard this morning, I am convinced there are many, many more we haven't yet discovered. The D, there is one antigen. Maybe multiple parts, but it is one antigen. And they are pushing 200 known published and there are many more on the horizon.

In terms of the fear of giving a true D-negative recipient anything other than true D-negative blood, I think the simplest way of handling that is to give blood that is

D-neg, C-neg and E-neg. It would be a very rare case that you would harm a patient if you did anything but that.

DNA testing is more time consuming, more expensive. It is prone to misinterpretation because of these multiple alleles and is not an improvement, in my opinion, over hemagglutination for the routine determination of ABO and D.

(Slide)

But when could we use it or could it be useful? I think it is useful to resolve ABO and D discrepancies, and particularly that they do not become an

FDA -- if I can say that in this room. It would not be an FDA reportable error if we can show that it is due to a genetic variant and not due to a technologist error or a reagent failure. As long as your local SOP says that it is okay, then it is okay.

ABO genotyping can be useful to distinguish an acquired phenotype for an inherited one without the laborious family studies that we have done in the past, and many RH phenotypes cannot easily be defined by serological methods, either because we don't have a panel of monoclonal antibodies or we don't have the appropriate -- the antibodies to test.

So, DNA assays may be used to define some and precisely match D and e in particular antigen status of the donor to the recipient. This is particularly true in the African Americans, and therefore, sickle cell disease becomes the major disease.

(Slide)

So, testing donors for Do antigens. We have a fair amount of experience at the New York Blood Center. The typing for Doa, Dob, Hy or Jos antigen negativity is notoriously difficult. The antibodies are weakly reactive. They are only available in small volumes. Our anti-Dob is in a tube. We have about this much, and you get your hands chopped off if you even go near it. Christine controls that.

The sera often contain other alloantibodies. It is very difficult and you can't rely on the crossmatch either. So, at the New York Blood Center we use PCR-RFLP manual method to select -- well, first of all, we select the antigen negative donors that we need for any given patient. So we are already multiple antigen negativity and then we do the RFLP to test for Doa and Dob.

The donors that we select that way survive better in the patients. The patients do better when we select serologically.

We also take donors and test them with anti-Gya and if they are weakly reactive -- if they are non-reactive, chances are we have an anti-Gya, but we haven't been that lucky yet. But if they are weakly reactive, we test them again by PCR-RFLP for Hy and Jos alleles, and this has provided us with a larger -- I'm not going to say larger, but a larger inventory of valuable donors that we need for certain patients.

So I think this is a clear case where for testing Do polymorphism by DNA analysis surpasses hemmaglutination.

(Slide)

Similarly, for the low prevalence antigens. In New York we have a very diverse patient population and many of those patients need blood that lacks Jsa or V/vs Joa or DAK antigens, and these patients usually have multiple other antibodies. And in the listing there I have given just four, but that is what we call the sickle flavor. It is a very common phenotype needed for African Americans.

The antigens, the Jas, the V, the S, et cetera, are very immunogenic and they are present on up to 20 percent of red cells from African American donors. So a few years ago, when we were clever and we found these combinations of antigen negatives in our African American donors, we were also immunizing patients to these low prevalence antigens. So now one in four or five of the donors are incompatible.

The antigens are not on the antibody screening cells or antibody identification cells mostly. The antibodies are not available to screen donors and

the crossmatch is not reliable. So we now have the big problem that DNA assays can provide a tool to resolve.

(Slide)

This was one particular case we had. It was a patient with anti-U, +C, +E, +K, plus V, S and Jsa. We had 95 eligible U-, C-, E-, K- donors, but of those only four had been typed and shown to lack both antigens. Twenty-seven were positive for one or other of the antigen and 64 had not been tested for either or both, and we just didn't have

anti-VS and Jsa available in sufficient volume to screen. It is very frustrating. The possibility of microarray technology to test donors can help us overcome these problems.

(Slide)

So, what are the pitfalls of DNA analysis? As any technique, there are technical, medical and clinical. I am not going to go into technical. The list is the same as the usual stuff.

(Slide)

Medical. There are probably two scenarios when the DNA and the hemagglutination test results may not agree. This is after recent transfusion, after stem cell transplantation and in natural chimerism. There has been a couple of cases in the public, in the newspapers recently, where peoples' blood type didn't match the typing of some of their tissues, and they were told in two cases that they were non-maternity.

One woman almost lost her children because of it, but they eventually found she was a chimera. And DNA results may differ somatic cells from

peripheral blood. It may not agree in a stem cell transplantation and the natural chimera. I mean, natural chimera maybe doesn't belong on the medical pitfall list, but I wasn't sure where else to put it.

So again, it is as important, if not more important, to always obtain an accurate medical history.

(Slide)

These are some of the other limitations of DNA assays. The result is not a phenotype. It will only ever be a analysis of that point of the gene, that SNP. It would never be the phenotype or what is expressed on the red cell.

It, therefore, can detect a grossly normal gene that is not expressed on the red cells. Now, this would lead to a donor being falsely typed as antigen positive and loss of a very valuable donor, usually the null phenotype. But it would not jeopardize the safety of blood transfusion to a recipient.

I think that certainly one should discuss and figure out when and how and to what extent the results should be confirmed by hemagglutination. And obviously, two approaches would be, if the reagent is available, do the confirmation, and then either that end or if the reagent is not available, just the crossmatch using a method that is optimal for the antibody and antigen in question.

I think another limitation is that not all polymorphisms can be analyzed. There is a large number of alleles that encode one phenotype, particularly ABO, RH and the null phenotypes, although the molecular basis is not yet known. Now, the large number of alleles, yes, we can eventually put those on a chip.

But the number of alleles giving rise to a phenotype tells me that

almost every individual with that phenotype is likely to have a different molecular basis, and there is a high probability that all the alleles in all ethnic populations are known. In fact, I would say they are not known.

(Slide)

Maria asked a question about IRB and I thought I would put this in just as a reminder. If we are doing tests for clinical purposes, you do not need IRB. There is no need for it at all. But since this is new technology, I think there is a very high probability that we are going to publish these results.

So, if you publish, then it is considered research. So you should consider IRB approval. It is much easier to get it up front than it is retrospectively.

Several things can dictate how complex that could be. If you are testing existing samples versus a sample that is collected specifically, if it is unlinked sample versus one that is linked or if there is risk or no risk to the person giving the blood, it would depend on whether it is exempt, expedited or a full review.

(Slide)

And as with all techniques in things, there is no such thing as black and white. Sometimes there is. Or usually, it is grey. So I think we have to think about and discuss and determine when we can use this technology and don't just use because it is the latest gadget and I want it or I want to be first on the block. I think we should be intelligent about how we use it.

(Slide)

And typing for DNA analysis. Some of the concerns is that the donor consent may or may not be needed. This would depend on the wording on the

donor registration form with regard to how much -- how the testing of blood group antigens would be performed. On our donor registration form we say that the donor is giving us permission to type their blood for antigens, negative or positive, and therefore, they don't ask what technique. If they do, we would certainly tell them.

There has been a lot of concern that such testing, as you have already heard from other speakers -- that the DNA typing would reveal unwanted information about the donor. I cannot stand here with my hand in my heart and say that genotyping for blood groups doesn't connect with a disease because of Kx and McLeod.

However, I can say that the typing we are doing gives exactly the same information as if we were doing it by hemagglutination. We are not looking for diseases. And the assays are not considered to be genotyping, but they are a procedure to antigen type by DNA analysis.

And we have discussed this with New York State, which is where I live and have to comply with their rules, and they say very strongly, and I have written documentation, that DNA testing for blood groups is not used to identify or diagnose a genetic disease; therefore, informed consent is not required. That was a huge step for us.

(Slide)

Antigen typing by microarray techniques. I thought I would put in some cost concerns. There will be some savings that may not be that obvious. The first one is that it doesn't precious expensive reagents. Maybe we just don't have it or it is just too precious to screen. It is really hard to put a price on that.

Manual testing and data entry are eliminated. There is a degree of efficiency. I think less skilled labor in terms of that subjective hemagglutination assay is not required.

We can use it to reduce the very time consuming testing that we currently do to detect alloimmunization in patients with autoimmune hemolytic anemia, and we wouldn't need to give RH immune globulin to fetuses that were shown to be D-negative.

We don't import -- from this country we don't import antigen negative blood very often, but it does happen. So, we can prevent that expense. There would be future transfusion reactions and we wouldn't need to treat those and pay for that.

It would be less likely to withhold transfusions, especially for those sickle cell patients, and thereby reduce the number of days in hospitals for them. So, improve their quality of life and reduce the cost of patient care as a general rule. And obviously, it will depend on how much the manufacturers are going to charge us, and I would say there is going to be probably a large expense in investigating all of the variants that come up.

If we find something different, we are going to want to pursue what it is and then will come to cost.

(Slide)

So, what are the possible uses for phenotypically matched blood as I see them? To match the antigen profiling of chronically transfused responders, especially those with sickle cell disease. With unusual RH phenotypes, there is going to be as many e variants as there are D variants.

And we can begin to match maybe at the genotype level and not even understand the phenotype. It may not be necessary.

Antigens for which there is no antibody. We have listed that same bunch. To test for Jka or Jkb in a patient who has been transfused to prevent transfusion reactions or even deaths to due antibodies to those antigens.

And each year the FDA, in the SHOT Program, which is Serious Hazards of Transfusions, in the UK received reports of patients dying from reactions to Jka or Jkb positive blood.

We could use it to match for DEL and D weaks. We could transfuse, if we wanted to be that clever, D weaks to D weaks or DELs to DELs, and we could save the true D negative for the D negative patients. As I said before, we have the rule of using C and E neg for the D negative patient.

And we can use it for patients with antibodies to high prevalence antigens. Christine made a good point earlier. If we had a sufficient number of donors that lack the high prevalence antigens and we could match the patient, then they would not make the antibodies and we would not later have to do all of those time consuming tests by hemagglutination to see if there was an alloantibody present underneath the antibody to the high prevalence antigen.

And we can also use it for patients with autoimmune hemolytic anemia. As I said, to prevent us having to do all of those adsorptions and how often do we do it and how are we going to protect the patient so that we could insure that there were no other underlying antibodies.

(Slide)

Other considerations that need discussion or decisions is to establish

of testing alleles for each antigen. In fact, for Fy, for instance, we always test for GATA and the nucleotide 265. But how many alleles do we want to test for any given antigen?

Can we test the donor once? I think it makes perfect sense if -- and it is a big if I think -- we have a simple, inexpensive way to positively identify the donor on subsequent donations so we can tie the one typing to that person.

Can we use the results without confirmation of hemagglutination? And when would automated DNA preparation and positive identification of a donor sample from the tube to the computer so that we are absolutely certain that we have the correct identification of that donor?

Another consideration is is it going to be a problem with chimeras. We think it is rare, but we don't know how rate it is. The testing hasn't been done. We have no way of knowing, but it is unlikely that the DNA from white cells would be different from red cells. It is possible, but it is very unlikely. So I don't think that is a problem.

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So in conclusion, I think we have tools available and hemagglutination and molecular biology are both very powerful techniques. They both have their uses and we should be intelligent about how we use them together to help the patient. Thank you.

(Applause.)

MS. KOCHMAN: And now we have Dr. Greg Denomme, from the Canadian Blood Services and Mt. Sinai Hospital, and as I mentioned before, he is coauthor on one of the books that all of us need to have handy.

Fetal Blood Group Genotyping

By Dr. Greg Denomme

DR. DENOMME: Thank you, Sheryl, and thank you for the invitation to speak, and a special thanks to Marion Reid, who I know very well and she put my name forward.

So, this morning what I would like to do is speak with you about one aspect of molecular typing in immunohematology, and that is probably the first application of the fetal blood group genotyping and it is a service at Mt. Sinai Hospital.

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This morning's objectives first is to just provide you with a short historical perspectives. I do predictive genetics. At least at the institution I am involved in we call it predictive genetics. The predictive in genetics I review paternal zygosity and fetal DNA genotyping.

I would like to review more closely some impact data. So, is the work making a difference? And then briefly -- sorry. But just a little bit more than you heard this morning; is to discuss fetal DNA in maternal plasma.

So here we have, back in 1964, the x utero blood sampling and transfusion of a fetus, and we have come a long way since then.

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So, a little bit about predictive genetics in hemolytic disease of the fetus and newborn.

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Prior to 1940/45 fetal deaths per 1,000 live births was in the order of four or five. And then as technologies and clinical applications were implemented in obstetrics there was a large drop in the number of deaths. So, Liley developed a technique where the supernatant of amniotic fluid was evaluated OD450 and a prediction of red cell destruction could be, and that is around 1960, but it took until 1994 before we were able to use the pellet that everyone discarded in a way to predict the fetal RH type.

As stated in some of the literature in your handout, Phil Bennett and colleagues in the New England Journal of Medicine provided a technique to determine a fetal RH type. In the same year Highlands Group in Blood produced a similar article.

Then in 1993 Dick Oepkes, from the Netherlands, had begun his graduate work on the Doppler studies in hemolytic disease of the newborn.

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Well, what are the sources of fetal DNA? Originally one source was chorionic villi. This is not a product of -- or a source of DNA of choice. As late as '91 limb deformities, a deformity called amnio bands, can occur with a higher frequency when chorionic villi are sampled. We actually looked at the cervical mucus plug. It turns out that it is a variable and not a reliable source of fetal DNA for a number of reasons.

Amniotic fluid taken some time less than 20 weeks gestation, say 17 and 20 weeks. It is easy to obtain a primary culture of amniocytes, but the cells in amniotic fluid are mostly dead epithelial cells and you will see where that has an

impact. And the risk of doing amniocentesis, the latest study is in 2004, is about a one in 300 risk of fetal loss.

And then, Fetal DNA and Maternal Plasma was first published by -- I believe it is Dennis Lo in Lancet.

(Slide)

So what are the procedural risks? Well, cardocentesis transplacental hemorrhage is high, especially if the placenta is anterior, antibody titre arising as a result of cardocentesis is very high, and fetal death in the order of three to 10 percent. At least in hemolytic disease of the newborn we are seeing these numbers more like three percent in various studies.

Amniocentesis done between 17 and 20 weeks gestation, a rise in titre are very unusual, as a transplacental hemorrhage if the placenta can be avoided. Fetal death, as I mentioned, about one in 300. And, of course, maternal blood as a source of fetal DNA. There is no risk in a placental hemorrhage, no risk to rise in titre and virtually no risk to the fetus.

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So, what would be -- the genotyping as of 1993 is that the father had to be heterozygous. That would be the first criteria. In other words, every is affected. The history would be women who would be considered a candidate for determining the fetal type. It would be those that have either a minor or severe history of hemolytic disease of the fetus or newborn, or the antibody titre would be moderate to high.

The amniocentesis was preferred less than 20 weeks gestation, but the work that we have done has often shown it is around 24 weeks. Maybe a little

bit further along.

When it is taken, the fluid is harvested and the bilirubin is estimated, regardless of what other tests are done. The cells are used as a source of genomic DNA and the fetal group blood genotype is determined. And antigen negative fetuses are returned to their primary care physician. Antigen positive fetuses remain within the tertiary care environment and a management strategy is devised.

(Slide)

Paternal zygosity I would like to review with you, because it is a little different than the remainder of blood groups. The remainder tend to follow standard mendelian inheritance, but RHD is a little bit different. We know that there are people who are homozygous for the D gene and the term when one of the chromosome ones have a D gene absent is they are hemizygous. And really, it turns out we are interested in the copy number of functional RHD genes.

(Slide)

This is an example of historically how RH genotypes were determined. This phenotype here positive for all the common RHD antigens. Possible genotypes are R1, R2 or R1r double prime within the Caucasian population. If we look at historical data, there is about a one in 10 percent chance that we are wrong.

As for C, E negative phenotype and an RH positive, either RO/RO or RO/r, and it is a 50/50 chance that the person is heterozygous.

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And Wagner and Flegel in 2000 determined the structure of the RH

locus, and quite readily it was obvious that a homozygous person had two copies of the D gene, a hemizygous person had one copy and what was important is that there was a presence of some material upstream and downstream of the RH locus, which was recombined and termed a hybrid rhesus box. And it is the detection of that rhesus box that became important in determining zygosity.

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Very quickly, Chiu, et al in Clinical Chemistry a year later used a somewhat quantitative way of determining RH zygosity by determining the gene dosage, and they also worked on a hybrid PCR that detected the central region of the rhesus hybrid box.

There are some confounding problems. One of the confounding problems is that a hemizygous person could have a functional copy of RHD and a pseudo gene, the most common being the pseudo gene with an insertion in exon four and a few other changes. Or someone could be homozygous for RHD and they have what we would consider a pseudo-hybrid rhesus box and that the downstream rhesus box had some unusual variations that would make it look like a hybrid rhesus box.

And a number of studies were done to try and examine pseudo hybrid or novel rhesus boxes and how the assay could be improved to improve the accuracy.

(Slide)

Well, what is the future of rhesus box analysis? It turns out that novel upstream and downstream rhesus boxes confound PCR-RFLP and the double amplification refracturing mutation analysis, which is the method of Chiu, would be

confounded by non-functional genes.

So, any non-functional gene will confound dosage, and so the compromise, at least the compromise that we are looking into, is two assays with a combined high specificity. Or an alternative would be a long-range high fidelity PCR that identifies the RHD negative haplotype outside the hybrid rhesus box.

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Now, a little bit about fetal blood group genotyping.

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The clinically relevant antigens that are typed at Mt. Sinai Hospital are RHD, E, c, e and C if the woman is RHD negative. Also, the KEL system; s is provided outside the fetal testing M or N. The Fy, Jk, HPA-1, 3 and 5 and a smattering of others.

The techniques preferred are PCR-RFLP, if it is possible. SSP-PCR is used, and occasionally when we have an orphan gene that we will only use once we would develop some sort of strategy that would involve sequencing around the polymorphism, and the best example is the Milton Berger, which we have done several years ago.

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The preferred phenotyping at Mt. Sinai is multiplex RHD amplification. It was described in Transfusion in 1998. This assay very readily will amplify RHD exons 3 to 7 and 9. What is obvious is that the RHD pseudo gene is very easy to see. We also confirm that the D pseudo gene is present by a specific assay for that.

We can also detect some partial D's, including D3, category D's

including DC3. Also, the C, D, e, s, now shown here, D6 type 1 and type 2, but we are also able to show that a weak D type 1 in a fetus would come up in positive R2/R2. So a double dose of D is positive, as is a single dose of the D gene. So it works quite nicely.

We followed the testing by testing the mother. It is obligatory RHD multiplex. We look for the pseudo gene and C, D, e, s. If that is positive in the fetus, we do multiplex. If we suspect a pseudo gene, we do the test for that. And up until the end of 2003 all of the results were repeated with cultured amniocytes.

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So, samples submitted for analysis. This is from April '97 to the present. Samples were tested either on direct amniotic fluid, so that would be genomic DNA harvested from the amniocentesis, or amniotic fluid plus cultured amniocytes. That is to 2003. And cultured amniocytes alone was about 15 percent of our population. In some instances referral hospital, instead of genetic labs, would send us mono layers for testing.

(Slide)

And here is a profile of the antigens that we tested. Still in 2006. This summary is 40 percent of our work is determining the D gene in the fetus, 20 percent K, and then C. It is always in combination with D, c, E or the remainder and then the rest are of lower frequency.

(Slide)

Lessons learned from sequence specific PCR, June 1997 to October 1998. We had a total of 14 amniotic fluid tests of which two gave a false negative when repeated using cultured amniocytes. And the assay that was used at the

time -- I am just using this picture for an example. In fact, the allele of interest, the K allele, was larger than the internal control and what we were having was high allele dropout that occurred in direct amniotic fluid.

Likely, a result of the quality of the DNA and we quickly dropped that assay and began to insure that the positive control for the presence of DNA was larger than the SSP primers. So, we use PCR-RFLP. This is an example of K/k typing. And insure that all of SSP-PCR D allele is smaller than the control, internal control.

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So, distribution of antigenic results. We had a total of 152 positive results, 131 negative and a total of 14 inconclusive, and this included inconclusive if the cultured amniocytes were tested. And it looks inconclusives could occur in any of the blood group systems. So it really was a function of mostly unable to get sufficient material. The cultured amniocytes may not have produced a cell line, and the remainder would be that it is not available and the direct amniotic fluid was inconclusive.

Two of them would have been prior to 2004 when we didn't detect a pseudo gene. So, if we detected a fetal RHD positive and the mother was RHD positive, we simply said that the test results were inconclusive.

I don't have the data here, but we tested the father when possible, and it would be likely close to 80 to 90 percent of all samples submitted.

(Slide)

Okay. What is the clinical impact? I think that is really the important part here.

(Slide)

Well, in the profile of patients submitted, these patients' baby was at risk of hemolytic disease. Some 40 percent had mild risks and received phototherapy at birth. About a fifth of them had moderate disease and they had phototherapy, plus an exchange transfusion at birth.

The severe, which was a quarter, had inter uterine transfusions and/or exchange in phototherapy at birth, and in extreme cases, which was a little less than 15 percent, they had severe disease and it included those fetal demise. There were three fetal deaths since 1991. But '97 is from our testing.

How did these arise? How did alloimmunization arise? A quarter of them -- almost a quarter are from abortion, and again, almost equal number from transfusion. We think that about 10 percent are missed RH immune globulin and a third we are really unsure of the route of red cell immunization.

(Slide)

Well, is the impact of predicting risk? We separated all of the tests that we had a result on antigen positive or antigen negative. We looked at the number of visits, and there were a number of clinic visits, the number of ultrasounds and the number of amniocentesis, and it is fairly obvious.

Once we find an antigen negative fetus, the number of clinic visits drops to four. So these women still come back. Although they are probably reassigned to their primary care physician, they come back for an ultrasound and Doppler. This is literally one quarter of the testing that is done in those fetuses that are determined to be at-risk.

The number of ultrasounds drop again by about a quarter, and

literally, we are looking amnios that run -- they have only had, on average, one amniocentesis, and that was the one to do the predictive genetics.

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Well, how about the number of requests per year and what is happening '97 through to 2003? We pretty well saw a steady increase of testing, and this included as we expanded the number of alleles or the number of typings that we could do. But there has been a sharp decline from 2003 on and this is the year that Dick Oepkes left Mt. Sinai Hospital and returned to the Netherlands.

And the department using a medial cerebral artery velocity Doppler to evaluate risk Doppler to evaluate risk, and I'm told that the best obstetricians are those that sit on their hands. And what we are seeing is a drop in amniocentesis because we likely have antigen negative fetuses in this group that they feel it is not worth the risk of doing the puncture.

(Slide)

Well, now a little bit about fetal DNA and maternal plasma.

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I took a summary here of a group of tests on fetal typing from 1998 through to 2005. The largest study by van der Schoot. This is, I believe, published in 2005, 1,200 women, and I couldn't get idea of the gestation. This is from a poster with a greater than 99 percent accuracy.

It included those that were mistyped on both sides. I think two false negative and three false positive. This has been presented in oral presentations at a number of places.

The controls were the SRYs, so the test for male fetus, plus insertion

and deletion, polymorphisms in DNA looking for something; that the father is different from the mother. Two of the publications are from the same group. So, a larger study population.

We are achieving close to 95 percent and above in determining fetal type, both included within hemolytic disease of the newborn and as a anti needle tool, but what is important here is to understand that there has been a 40 percent overall reduction in invasive testing.

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So my closing remarks. In the early '90s, when the RH locus, the RHD gene was first cloned, immediately we recognized the test could be used to determine risk for hemolytic disease of the fetus and newborn; D and quickly thereafter C, E and so on.

By 2000, as the RH locus organization was worked out, we now have a very good test to determine paternal inheritance. This is the only real conundrum that we have to worry about that serology can't tell us with a high degree of accuracy, and then there is a long list that Marion Reid had provided to you.

I think what we can conclude is that fetal blood group genotyping is a reliable predictive genetic test and it makes a difference in the clinical arena. Rhesus box and RHD gene dosage have a high specificity. We do require additional genetic information, including probably RHCE haplotypes.

Fetal blood group genotyping has an impact on the management of hemolytic disease of the fetus and newborn, as you have seen, and eventually fetal DNA in maternal plasma will replace fetal RH typing in hemolytic disease of the fetus and newborn. I don't think there is any question that given even the small risk

of amniocentesis, if we can get fetal DNA in maternal plasma nailed down for RH, I think that will make a big difference.

And the role of fetal DNA in maternal plasma for postpartum and antenatal prophylaxis is really still up for discussion. And I believe that is it. Okay. Thank you very much for your attention.

(Applause.)

MS. KOCHMAN: And now, Dr. Connie Westhoff, scientific director of the American Red Cross and adjunct facility faculty at the University of Pennsylvania is going to talk about Rh complexity.

Rh complexities: Serology and DNA Genotyping

By Dr. Connie W. Westhoff

DR. WESTHOFF: Thank you, Sheryl, and thanks to the FDA and the NHLBI for sponsoring the workshop and for inviting me to participate.

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When Sheryl talked to me about doing this talk, one of the things she mentioned was that she wanted the European experience presented first, followed by the U.S., Canada and South American. So my goal today is to give you a feel for the U.S. experience, with the caveat that this is the American Red Cross referral experience in our institution.

And hopefully, Marion and Christine will agree that it might reflect some of the things they are seeing also. What I want to cover today is why serologic D typing is not always straightforward, and I want to bring to your attention that C and e typing isn't always straightforward in the U.S. to kind of summarize the problems of how they present, what are the clinical implications and

give you a few examples of the cases we have seen.

And then lastly, I would like to finish with our perspective at the American Red Cross for the role of genotyping in transfusion medicine going forward, both currently, because currently we do feel there is a role for genotyping in transfusion medicine; we have been doing this for about three years, and certainly expansion in the future.

(Slide)

So, I would like to begin with the question of why is D typing sometimes problematic? Well, in the U.S. it is because of a large number of variables, which have been mentioned by some of the other speakers in our serologic testing.

You all know there are multiple methods in use out there. Hospitals use tube, solid phase and gels. Some perform the AHG test or the IAT for weak D. Others do not.

The other issue is that we are often using different reagents and that these reagents, these monoclonal reagents, contain different IgM clones, and these can react differently, especially with our weaker variant D antigens. And currently, the FDA only requires that the reactivity with these three be specified at the manufacture level. So, this really results in the D typing discrepancies.

Layer on that the variability of D protein. It looks like I have to change that 120. Each time I give the talk it goes up. Dr. Flegel informs us 180. We are in a race with ABO actually to see who has the most number of variants.

But these are all due to changes at the DNA level from conventional sequence, and here is a flavor of all of the weak D's; 53 partial D's, about 45 DELs,

et cetera.

(Slide)

So, I would like to just summarize again, as Dr. Flegel has done very admirably, what are these variations and how do they react with our U.S. clinical reagents. We are just beginning to get a handle on that.

Now, I would like to remind you that the majority of D positive donors and patients do have a conventional D gene. In other words, the standard common sequence. But, of course, we have all of these variables from weak D, which we used to call DU, whose incidence really isn't well known in the U.S.

Certainly there is the European experience, we know there are wide population differences and we only identified the weak D or defined it in the U.S. as requiring the indirect antiglobulin test to detect these cells. And really, it depends upon the reagent you are using also because different monoclonal reagents will detect a sample as weak D or strongly D-pos.

DEL has been in the press a little bit more lately than usually. These cells have very, very weak expression. So you can only detect them if you adsorb and elute anti-D and enhance the EL classification.

In partial D we all were taught in training that these were mosaics that were lacking part of the D protein or missing part of the D protein, and these type with our U.S. reagents is D positive, but the problem is they make anti-D.

Another category that you might not be aware of is those that have been found recently to be actually just D epitopes that are actually expressed on the ce protein. So, there is no D protein in the membrane, but there is an epitope that mimics or is identical to something that is reacting with the monoclonal anti-D

reagent.

And I will talk a little about these because they do cause D typing discrepancies that we see in our laboratory.

(Slide)

So, just briefly again. On the Weak D's most of the work on this has been done by Wagner and Flegel. Again, they showed that these were single gene mutations and they were nicely located intracellular or predicted to be intracellular or cytoplasmic. And so, this we thought explained why the effect was on the quantity of protein, the way the protein went and packed in the membrane, rather than on the D epitopes on the surface.

And we had always historically felt that these were people who were not at risk of making anti-D, and now we know there are some exceptions. But here are shown some of the 53 different weak D's and where the mutations lay within the membrane.

So, the problem with our weak D is that reactivity of weak D is variable with different monoclonal reagents and with different techniques. Many of these can be three-plus, but some can very weak, plus or minus, or even missed by the indirect antiglobulin test. In our experience what we see is a lot of weak D type twos in the U.S. are being missed, but they are being picked up gel testing, and I will talk a little bit about that later when I summarize the things we have seen this year.

(Slide)

DELs, of course, as we have identified, they are D negative, including the IAD test. There are several different mutations that cause the DEL. They are

more common in Asians. Being D negative is very rare in Asians, but when it does occur, a third of those are thought to be carrying a DEL gene. It is nice in that all of these are also C positive, and when you find this mutation in Caucasians, they are also C or E positive.

Although they recently have been in the news because they can stimulate anti-D in recipients, most of us would agree that they should be D positive donors. But as has been pointed out by other speakers, if you do a C or e typing on a D negative donor, that would potentially screen these from the donor pool.

So those of you that were around in the old days when they used to use a D, anti-D, D, C, E reagent to screen donors, I guess what goes around comes around.

(Slide)

So, partial D's. Again, I mentioned that the type is D-pos. They make anti-D. The problem is here you don't detect these until they make the antibody. And nicely, these mutations map to the extracellular surface. So we have a nice clear picture that these probably alter D epitopes and explains why they make anti-D.

Now, some of the partial D's are due to these single mutations. So all we have to do to find them is target a single site, and these are noted here. But by far the majority of partial D's are due to a gene conversion event where part of the CE gene actually replaces part of the D gene. So, these are new hybrid proteins.

And so what happens in these is that you are actually altering D epitopes and at the same time creating new antigens. And so, this finally explains why this blood system is so polymorphic. You have got two genes sitting very

close together undergoing gene conversion and rearrangement events between them, and so we have an unlimited possibility for diversity here.

Evolution and action is one of the things that we see here at the RH locus.

(Slide)

So the last category are the D epitopes that are expressed on RHCE, and I want to spend a little bit more time on these. I will remind you that these do not have a D gene. In the Crawford, which are found in Blacks, they have amino acid change in exon 5. That is a D specific residue. And in the D-HAR, which are found in the Germany population, Germany immigrants in the U.S., the exon 5 of the CE gene has actually been replaced by exon 5 of the D gene. So there you get the D epitope.

And these D specific amino acids in a CE protein on the surface of the membrane strongly react with some of our monoclonal anti-D, but not all. So, in the U.S. these are the major causes of the D typing discrepancies that we have referred for D gene investigation.

(Slide)

So, let's look a little bit about our licensed reagents in the U.S. You all are familiar that we have four reagents licensed for tube testing, one for gel and only two contain the same IgM clone, the Immucor Series 4 and the Ortho gel.

So again, like I mentioned, these clones can react differently with variant D antigens, and it is the IgM component of the reagent that is doing this.

(Slide)

So, here I summarize our experience with looking at the difference in

reactivity of D-HAR and Crawford red cells with these different reagents, and as you see here, if you have a D-HAR cell, it is strongly positive with all except the Ortho bioclone reagent. It is completely negative.

And again on the Crawford side, the Crawford strongly positive with the GAMA clone three to four plus and completely negative with the other reagents. And so I believe that these two phenotypes actually, since they are completely non-reactive with our old human source monoclonal anti-D, is really what has contributed to some peoples' perception that we have a lot more typing discrepancies since we went to monoclonal reagents and often kind of whine for the good ole days with the human polyclonal.

But I think these two are, in our experience anyway, the discrepancies.

(Slide)

What is the clinical significance of, for example, say Crawford? This is a case that we just saw a couple of months ago. It was an 81-year-old African American woman. In 2003 she typed three-plus D-pos, and she got three units of blood. She came back to the same center in 2006; severe amenia and active GI bleed.

She again typed as a D positive with a negative antibody screen and they gave her three units of blood, followed a day later with a couple more. Within eight or nine days here she had a strongly positive DAT, her hemoglobin had dropped down below original and she had an elevated bilirubin. So, undergoing delayed transfusion reaction in both the eluate and the serum showed that she had anti-D.

(Slide)

So, how did these present? When we get calls about discrepancies in reagent, we always counsel people that if your GAMA reagent is strongly positive and Immucor or Ortho bioclones are negative, this is a hallmark for the Crawford phenotype.

(Slide)

People often ask us for recommendations on what typing reagents they should be using, and we feel that as patients and recipients these -- both the D-HAR and the Crawford should certainly be typed as D negative for recipients. As recipients they don't have the D gene. They don't have the full D protein, and they have both been shown to make anti-D and HDN also in D-HAR.

So, for a patient typing reagent we always recommend -- we prefer the Ortho bioclone, whereas on the donor side we would recommend the GAMA clone. But again, how often are you going to see things like this?

Well, the Crawford is estimated to be 1 in 900 in the southern U.S. by John Moulds and colleagues. The D-HAR, there is a larger pocket of Germans in the midwest known to be D-HAR. In our experience though Crawford certainly represents the largest number of samples that we get in for D discrepancies.

(Slide)

And this just summarizes our data from just this year on what kind of things are referred to our lab, and for D typing discrepancies Crawford really is nearly 50 percent of the cases. We also investigate a lot of D-pos with anti-D, and we also do zygosity testing.

(Slide)

So then what is our goal here in the U.S.? On the donor side anyway? Our goal is certainly to label all donor red cells with some D antigen as D positive, but our problem is that at weak D some of these are missed even with the indirect antiglobulin test, and like I said, in our experience we are seeing that on the weak D type twos. And the other problem is the DELs.

If you have a large Asian population of donors you are drawing, you might be concerned about that because these all type as D negative and both of these can stimulate anti-D in our D negative patients. But important to remember, like I mentioned before, all DELs to date and the majority of weak D's are inherited with a C or a E phenotype. So you can simply remove these from your donor pool by serologic typing for C and E.

But I think the basic questions haven't been answered yet about our policy in the U.S. Will we accept absolutely not anti-D production in any donor of any age? We certainly don't want to do it in girls and women of child bearing age, but at the same time we accept the risk of

anti-KEL, which has caused hemolytic disease of the fetus and newborn and anti-c in the U.S., whereas in Europe many honor KEL and c in females in child bearing age.

So, is this then an inconsistent policy? Those are the kinds of discussions that we need to begin to have.

(Slide)

Now, on the patient and OB side our goal, of course, is to detect any of those at risk for anti-d. And again, most weak D are not at risk for anti-D. But as we heard this morning, there are exceptions to these that we label as weak D 11,

15, 4.2 that do make anti-D. And I think the jury is still out on how many of our weak D's can make anti-D.

We have certainly seen what we think as allo anti-D in weak D type twos. But again, the problem is when you are investigating these cases many times the antibody response is auto before it becomes allo. Any of you who have worked on the serologic bench for a long period of time, when a patient first makes an antibody the specificity isn't sharpened up and it often presents as an autoantibody rather than alloantibody. So, I think the jury is still out on weak D's.

But the problem, of course, is that our serologic tests cannot distinguish weak D from partial D and, of course, weak D mutations may also have altered epitopes. We don't know that.

(Slide)

So, would DNA genotyping then solve this D typing problem in the U.S.? I think this is more complex than just a change in methodology because how do you act on the results then? If you are going to get a response back that is weak D type six, weak D type seven, et cetera, we are going to have to have a policy on what to do with all of that information.

And there has been a move to drop the weak D category label and drop the partial D category label and just call it a variant D, because that is truly what it is, because it is felt that weak and partial labels are trying to communicate some kind of clinical path to follow.

But I think they have useful labels. They are useful labels to at least say most weak D don't make anti-D; many partial D do make anti-D. So at least it gives us a category to put people in.

I mean we all know if we treated all variant D's as D negative, that would be a significant burden to the D negative donor pool. And so what we really need is additional data following up the D-pos who make anti-D, following up the D negatives who have received a transfusion and make anti-D so that we can know experimentally which D changes generate new epitopes. At least clinically in our patients, and we certainly don't have the experimental systems now set up to answer these questions.

And again, that big goal is will have any anti-D produced in any patient? You see lots of institutions that give RH immune globulin to 67-year-old males who get RH positive platelets, and is that an appropriate use of the products? So, we need to have these kind of discussions.

But what it is going to take, certainly genotyping for DNA, is a high throughput platform. In the last three years we have done a lot of gene sequencing and because so many regions of the gene need to be sampled, and it is also going to take some complex algorithms for interpretation.

(Slide)

So, in the last few minutes I would like to switch to CE. I used this summary of the problems we have seen this year to point out to you more than D we actually see more CE variant typing problems, patients with antibodies, et cetera. So, what is happening here? When is the serologic C typing not straightforward?

(Slide)

So what is happening here? When is the serologic C typing not straightforward? Well, in African Americans and in some of our Hispanic groups

and mixed ethnic backgrounds there is a specific D/Ce/D hybrid gene. By the way, I have the number. It is three to seven. I have the hybrid gene structured wrong there. It is exon three to 7 that are replaced. But this hybrid gene is a D negative, but confers to the red cell C positive phenotype.

So, individuals that are carrying this kind of RH haplotype at the D locus and CE locus, they type as C positive. It is coming from the D, C, E, D gene, but they can make anti-C because it is different than the conventional or Caucasian C haplotype.

But this D, C, E D hybrid gene is also linked to a variant e gene. So they are also at risk to make anti-e. And unfortunately, this RH haplotype is prevalent in our sickle cell populations, estimated to have a prevalence of about 22 percent in African Americans and underlies some of the alloimmunization and difficult incompatibilities at the RH locus for our sickle cell patients.

(Slide)

Now, the other issue here is when serologic e typing not straightforward. Again, in those same groups, Hispanics, African Americans and mixed ethnic groups. I show the conventional c gene as the pink tin boxes, but there are many different genes in these populations and they all encode altered expression of e because that is the only marker we can use on these.

We certainly know they convey different antigenic epitopes on the membrane. These are often known as HRS or HRB, and this isn't a complete list. But the point that I want to make is that there aren't serologic reagents available to detect these different variant forms of the CE protein, and so, that suggests that genetic matching is something that is going to really help here because all of these

type a e-pos. So, serologically you can't detect them and them make anti-e or anti-c and e. Again, prevalent in our sickle cells patients.

(Slide)

And to layer another complexity on top of that, it is that inherited with these altered CE genes -- and again, this isn't all of them -- they are often inherited with that altered C I showed you about. They are also often inherited with altered D. So, what you have in these sickle cell populations is the potential to make anti-C, make anti-D and to make anti-e or CE. So I think the surprise is that we do do as well with our sickle cell patients as we do.

I think when we start transfusing them as babies their not quite as immuno competent. So there is suppression going on. But what we do see when they do make antibodies is this kind of profile, and this then is a very difficult situation to try and transfuse over.

(Slide)

So, our view of the role for genotyping in transfusion medicine currently is the same kind of laundry list or litany that Marion and Greg both showed you. We are using it to type multiply transfused patients, et cetera. But number three, we are using it to resolve those reagent typing discrepancies, and I think it is important that we use molecular techniques to show our shortcomings in our serologic reagents and actually use that knowledge to design future reagents.

We are also using it, like Greg talked about, to predict fetal risk. It also helps very much in resolving antibody identification. Is it allo or auto? And we are finding out at the gene level oftentimes it is allo, not auto.

And a real important point that we think is to begin providing

compatible units, especially for these sensitized sickle cell patients that are carrying variant D, C and E antigens.

(Slide)

But going forward, how do we do a better job? For our donors, if we have high throughput screening, we would like to consider screening for antigen negative units in this high throughput method by DNA based testing, but also do a serologic test if a reagent is involved, and this would provide us a time of overlap when we would have an opportunity to validate the genotyping and to detect any exceptions in different populations.

And certainly our thrust is for our patients with sickle cell that are in these antigen matching programs, because with the STOP trial, as Marion mentioned, this is one population in which transfusion is bound to increase. But at the same time, this variant C, e and D problem makes some of these recipients almost impossible to transfuse, and that is a lifelong sentence for that sickle cell patient.

So, we feel genetic matching up front or at least genotyping up front -- and I'm calling this genotyping because at the current time we are sequencing all of these genes to detect the polymorphisms, both in D and C, e, and we think we need to predict those at risk. We can predict those at risk for production of this high antigen antibodies so that patients that are homozygous for variant C, e and D can be identified and transfused accordingly.

(Slide)

So, for the future we see integration of DNA based assays certainly into blood bank and transfusion medicine as genomics moves forward and is

applied to diagnosis and treatment, and it is not too early to start thinking about a potential antigen matching program. Whether it would be 15 or 50 antigens, it is time to start thinking about what patient population this would be appropriate for; those needing long-term transfusion support and at what cost.

Or, at what savings. Because it certainly would impact our workload to have a walkaway system, and any time we can decrease sensitization and decrease the amount of referral antibody I.D. work needed with our short workforce, that certainly would be a cost savings.

But at the same time we have to look at the other side, which a donor management inventory issue. You could imagine a computer deciding what genotypes were needed in the blood bank and actually doing the calling of the specific donors, but you would have to completely change I think the thinking about the management of the donor inventory if you were going to match for 15 or 50 antigens in many different patients. But again, it is not too late to start thinking about that.

So in closing, I just want to say my vision right now of blood group typing is that we have got real power here in the combination of both techniques, a phenotype and a genotype and so our focus should not be to prove or decide if one method is superior or is ready to replace the serologic typing, but our focus should be to use each to strengthen the other, because the shortcomings of our D typing reagents can be revealed by doing the DNA testing.

(Slide)

So, use this knowledge to improve our reagents and at the same time the shortcomings of the SNP testing is revealed by our serology. So to use that

knowledge then to design additional or better SNPs.

(Slide)

With that, I would like to close with acknowledging my laboratory, who is busy sequencing these genes, and without their help and input I wouldn't be able to tell you about our experience. So, thank you.

(Applause.)

MS. KOCHMAN: I have us scheduled for one hour for lunch. Since there is a cafeteria right downstairs, I am hoping we can get everybody replenished in that hour. Like I said, they really would like us to be able to finish up by 5:00 today. So, we are going to hold questions from the three speakers that just spoke until this afternoon. So, if you could all be back by 1:30, that would be great. Thanks.

(Whereupon, at 12:35 p.m., a lunch recess was taken.)

A F T E R N O O N S E S S I O N

(1:39 p.m.)

MS. KOCHMAN: Okay. We are going to get started again. I think there are a few stragglers, but they will make their way in.

I would like to give Christine a more proper introduction this time now that my brain is in the same room as my body. Christine Lomas-Francis comes to us from the New York Blood Center, and as was mentioned when I introduce Marion, she is a coauthor on the Blood Group Antigen Facts Books with Marion Reid.

She has more than 50 publications, and my first introduction to Christine was when she was at GAMA Biologicals, since I do work more with the reagent manufacturers than I do with the users.

But I would like to have Christine come up now and give her talk on the Kidd Blood Group System. Thanks.

The Kidd Blood Group System

By Christine Lomas-Francis

MS. LOMAS-FRANCIS: Thank you and hello again. I hope you all had a wonderful lunch. I am going to be talking to you about the Kidd Blood Group System, and from the previous speakers you will all be aware of why the Kidd system is important in transfusion medicine, and therefore, it deserves a place in today's discussions.

(Slide)

I am going to give you some information first that is background information and then talk about the different antigens, phenotypes and antibodies within the system, briefly touch on the molecular genetics and focus on some of the

clinical challenges that the antigens or antibodies of the Kidd system have given us. And then, conclude with the value of DNA analysis for patient care and for the provision of antigen negative blood.

(Slide)

At the antigen level the Kidd Blood Group System is actually very simple. There are three antigens. Jka and Jkb are antithetical and polymorphic, and Jk3 is an antigen of high prevalence. The antibodies are clinically significant, but the antibody detection and identification of these antibodies is notoriously difficult.

And as Marion has already said this morning, there are several deaths due to incompatible transfusion reactions reported each year to the FDA and to her international bodies who monitor transfusion safety. We now know the molecular basis of the Jka and Jkb polymorphism, and that is why we can talk about the system today.

(Slide)

There are only four phenotypes. Just a couple of things to highlight. One is the high prevalence of the Jkb negative phenotype in the Black population, and the other thing is that the system has a null phenotype, the Jka-b- phenotype that is exceedingly rare in less populations, but is found with greater prevalence in Polynesians and in Chinese populations, and actually also is slightly more common in the Finnish population.

(Slide)

The antibodies of the Kidd System are stimulated generally by transfusion or by pregnancy, although there are a few examples of non-red cell

immune antibodies that have been identified. The antibodies, as I have already said, have caused transfusion reactions. They have caused clinical hemolytic disease of the newborn, and they can be hemolytic both in vivo and in vitro. And they can be autoantibodies, which adds a complication to the whole system.

(Slide)

Anti-Jka is more common than anti-Jkb. Both antibodies have caused severe transfusion reactions, some of which are fatal, and the reactions are immediate hemolytic ones. But the antibodies are also regularly associated with delayed hemolytic transfusion reactions, which again may be severe with oliguria, renal failure or death.

One of the perhaps surprising facts is that at least one third of all delayed hemolytic transfusion reactions are attributed to antibodies within the KEL system. I hope that by the time I'm finished today with this talk you will have more ideas, if you didn't know already, why that is so. Anti-Jka is produced with the null phenotype; with the Jka-b- phenotype.

(Slide)

The Kidd antibodies can cause hemolytic diseases in newborns. The antigens are developed early in the fetus. Jka has been shown as early as seven weeks, but certainly the antigens are there by 11 weeks within the fetus.

Occasional cases of hemolytic disease have been severe, but many of them have actually been cases of only mild HDN that required phototherapy or no treatment at all, which may be a little surprising and there are various theories that have been generated about that. But that is not the focus of this talk.

(Slide)

The challenge with Kidd antibodies is the fact that potent antibodies are actually the exception and to the rule. So they are difficult to detect and sensitive methods are needed, and these methods may be the antiglobulin test with enzyme treated panel cells, column agglutination technology, solid phase or even polybrain.

To make it more difficult for the identification, the antibodies are usually found in sera containing other alloantibodies. The antibodies also may not react with panel cells that have a single dose of the corresponding antigens. In other words, the antibodies demonstrate dosage.

And then, to confound that even more, some of the antibodies don't react with all panel cells that have an apparent double dose of the antigen, and that is probably explained by the fact that those panel cells may not actually have a double dose of the antigen, but may have a hidden silent allele that is difficult to determine serologically.

The antibodies often deteriorate in vitro and they fall ball detectable levels in vivo, and thus, they may escape detection in sensitized patients resulting in a patient having an anamnestic response to transfusion of antigen positive cells that were perfectly compatible in the crossmatch.

(Slide)

At the molecular level the Kidd gene or the gene encoding the Kidd glycoprotein has been cloned. It is a gene of 11 exons. Exons 4 to 11 encode the mature protein, and the gene is located in the long arm of chromosome 18. And if you care to, you can access the sequence in the Gene Bank.

The glycoprotein encountered by the Kidd gene is a urea transporter,

ultimately referred to as hot 11, and it is present on red cells and also on endothelial cells of the phage erector and the kidney medulla. And the Kidd glycoprotein is a transmembrane protein, which is consistent with it having a transport function.

(Slide)

The molecular basis of the Jka, the polymorphism, is determined by the nucleotide at location 838. The presence of "G" encodes Jka and Asp280, whereas when an "a"is present, then the Jkb antigen would encoded and --- would be present at a --- residue 280.

In addition to that mutation there is another change at nucleotide 588G change, but this is a silent mutation. And furthermore, there is a polymorphism within the three prime end of entron nine of the gene.

Now, the 838G in the Jka allele introduces a restriction enzyme site, and this is what can be used for DNA analysis and for prediction of antigen expression.

(Slide)

The molecular basis of the null phenotype, the Jka-b- phenotype, has two different genetic backgrounds. We have homozygous inheritance for a silent allele, and at this point there are eight null alleles that have been identified. These alleles each have slightly different mutations that result in the null phenotype and the changes include mutations within the acceptor or donor splice site, genomic deletions, nonsense or misense mutations.

In addition to the inheritance of two silent alleles, the Jka-b- phenotype can also be caused by the inheritance by a dominant inhibitor gene that

is commonly referred to as In (Jk), and the gene that is responsible for this suppression of antigens has not been identified.

The most common silencing mutations for the Jka-b- phenotype are both on a Jkb allele. The one that is prevalent in Polynesians and in Chinese is a splice site mutation within intron five, which results in skipping of exon 6 and loss of additional exons and loss of the glycoprotein within the membrane.

And then there is a mutation that is found commonly in the Finnish population, which is a mutation at nucleotide 871, a T to C in exon 9, which encodes Ser293 to be changed to a protein. And so, based on the fact that we have at this point in time eight null alleles, to identify Jka-b- donors, multiple SNPs would need to be tested for.

But if you know what is more prevalent in the particular population that you are testing, then you could actually select procedures to define or to detect some Jka-b- patients with regard to donor testing.

(Slide)

I took this quotation from Issitt and Anstee because I think it sums up more of what I am going to talk about in a moment. They have written that the in vitro detection and characterization of these two antibodies, referring to anti Jka and Jkb, can be among the more difficult tasks that confront blood bankers. There are a number of reasons for the difficulties and many of them also contribute to the fairly regular role of the antibodies in causing delayed hemolytic transfusion reactions.

And I think the statement sums up beautifully what I was trying to show you in my earlier slides.

(Slide)

So, we are looking at DNA as an alternative way to test for Kidd antigens, and we are doing so because hemagglutination for Jka and Jkb does have its limitations. As for all of the other systems it is not suitable for testing multiply transfused patients, it is not suitable for testing the many patients whose red cells are coated with IgG, and hence, test positive in the direct antiglobulin test.

We do have monoclonal anti Jka and anti Jkb, but the directions insert says that the test is invalid if the red cells have a positive DAT and if they react with both antibodies, and we also have the possibility that antigen sites can be blocked with specific Jka autoantibodies.

We do have methods to remove IgG coding from the red cell, but these are not always successful. Hemagglutination also isn to suitable to determine the fetal Kidd type, and hemagglutination is not always adequate to screen for antigen negative donors, because potent antibodies are limited and expensive.

And the production of monoclonal anti Jka and Jkb has, unfortunately, not yet lived up to expectations, because we have only one FDA licensed example of anti Jka and Jkb.

(Slide)

So, based on what I have said in my previous slide, at the moment we would say that DNA analysis is superior to hemagglutination for testing multiply transfused patients, testing patients whose red cells are coated with IgG, to predict if an antibody is an allo or autoantibody, to screen for antigen negative donors, to determine zygosity on reagent red cells so that when we have an apparent -- we

have a cell of an apparent double dose of an antigen on a panel, we know that it is so. And also, to determine the fetal genotype and to predict thee phenotype.

(Slide)

We quite often are challenged by Kidd antibodies, and I want to share with you some cases that we have seen where DNA analysis has been exceedingly useful. The first one was a few years ago where the patient was in the emergency room with signs of hemolysis, life threatening anemia / DNA analysis / JKA/JKA / allowed for an appropriate course and the patient, under the circumstances, was incoherent.

Anti Jka had been identified previously and the patient had been transfused two units of Jka negative red cells. When the sample was tested in the immunohematology lab, the red cells appeared to be obvious for Jka and mixed field.

So, the interpretation that one would think of instantly is that we are dealing with a transfusion reaction because another hospital gave some Jka positive red cells, because records aren't always available and patients don't always remember, particularly when they are incoherent, if they have been at another hospital.

But we took the sample through for DNA analysis and actually found that the patient was Jka/Jka, and so the interpretation in this case was that the patient's red cells would be expected to be Jka positive and that the hemolysis was being caused by an auto anti Jka. And this then allowed for an appropriate course of treatment based on those results.

We, in our testing, have found a surprising number of autoantibodies,

particularly auto anti Jka, and so, whenever we identify a Kidd antibody, we are very cautious before we would say that it is an allo or autoantibody, if we cannot determine the patient's Kidd phenotype.

(Slide)

So there was a study that was reported at the ISBT meeting this September, a study that came out of Germany. The authors were Adam and Klutchner, and they looked at a number of patients who had been recently transfused or with signs of hemolysis or unexplained anemia. They tested the red cells, --- antiglobulin tests, performed crossmatch and also looked at the autologous control. And then they made alleles from red cell samples that were DAT positive and/or had a positive autologous control.

And by doing this within this patient cohort, they found 24 Kidd antibodies, 22 of them anti Jka and two anti Jkb, and the majority of these antibodies were not detected by retrospective testing of the patient sera. Now, 17 of these patients had required further transfusion, and when they looked, only 1 of the 17 sera was weakly reactive by --- agglutination technology with one of four Jka+b- red cell units in the crossmatch.

And I'm assuming that they cross matched these units before they had figured out that they were Kidd antibodies in the plasma. And these authors found that column -- sorry. That solid phase was actually more sensitive than column agglutination technology.

(Slide)

Well, given that scenario, can we improve the picture? These authors had 10 patients out of the 24 that had a mild delayed hemolytic transfusion

reaction. Fourteen patients had delayed serological transfusion reactions. So, the question is should all patients at this point have their Kidd phenotype determined? Should all transfused patients receive blood that is Kidd type specific, keeping in mind Insett and Antsee said about the difficulty of detecting the antibodies, and also keeping in mind the fact the number of patients that have delayed hemolytic transfusion reactions and animistic responses.

And now that we have DNA based assays, these, unlike the hemagglutination based testing, has the potential to allow us to type the patients so that we then can transfuse type specific blood if we are dealing with a multi transfused patients.

(Slide)

Again, the question of autoantibody versus alloantibody. We had another case just recently in which the patient was sent to us with an identified anti Jka, and the patient had been recently transfused. Two of those antibodies had been randomly transfused before the antibody had been identified. And then after that another two units were given of Jka negative blood, but there were signs of hemolysis and the patient's red cells were positive in the direct antiglobulin test and all crossmatches were incompatible.

In the immunohematology lab anti Jkb was identified in the sera and in the allele. But in addition to the anti Jkb there was also a weakly reactive autoantibody. We had two sets of patient samples, one that had been drawn after the initial two transfusions, and that sample typed 2+ with anti Jka and microscopic with anti Jkb.

And then the second sample, which had been drawn after the

transfusion of the two Jka negative units, that sample type microscopic with the anti Jka and 2+ with the anti Jkb. So, the complete reverse. So, we scratched our heads and we said, what on earth is going on?

(Slide)

The possibilities are that the anti Jka is an autoantibody or was incorrectly identified, or the anti Jkb is an autoantibody or that it was incorrectly identified. And at this point transfusion of Jka-b- cells, which would be ideal, is, of course, not an option, because nobody has any of those to spare.

The DNA based analysis showed that the patient is homozygous for Jka and that the patient's red cell phenotype would be predicted to be Jka+b-, and thus, the anti Jkb is an alloantibody and the hemolysis is likely to be due to the anti Jkb.

The scenario for some of these patients, if we are not able to figure this out, I think could be pretty awful.

(Slide)

So, in conclusion, DNA based Jka and Jkb antigen determination has the potential to enhance transfusion safety. That is because we can use it to predict the Kidd phenotype of patients without interference from transfused red cells or from IgG coding on the patient's red cells. DNA analysis allows transfusion of blood for Kidd -- I'm sorry. I tried to finish too quickly here.

DNA based determination does allow transfusion of blood that is matched for Kidd phenotype, thereby preventing several transfusion related deaths each year, many transfusion reactions and the associated trauma and also would alleviate the need to withhold transfusion.

It should make it possible to test donors without a concern over lack and expense of typing reagents, and it will also make it possible to type red cells to be used for antibody identification panels to insure that the persons of null alleles will not weaken antigen expression of apparent double dosed phenotype cells that are included on the panel. I thank you for your attention.

(Applause.)

MS. KOCHMAN: And now we have Dr. Lilian Castihlo. She comes to us from HemoCamp in Brazil, and she is going to speak, about the Duffy System. Thank you.

The Duffy System

By Dr. Lilian Castihlo

DR. CASTIHLO: Good afternoon. I would like to thank Sheryl, Maria and Marion for this invitation. It is a pleasure for me to be here and an opportunity to talk about our experience in the genotyping with you.

(Slide)

I will try to go over in this presentation what is currently known about the system genotypes, the applications of different genotyping and our experience in Brazil with the Duffy genotyping --- the transfusion of Fy(b) positive red blood cells in complicated situations to Fy(b)neg patients with GATA box mutation and the benefits and limitations of the genotyping.

The Duffy gene is located in chromosome 1 and is shown to have exons distributed over 1.5 kb of gDNA. There are two gene products. The major is of 336 amino acid and is called beta, and the minor is also 338 amino acid and called the alpha.

Like a protein Duffy is expressed the --- cells --- column, lungs, kidney, thyroid and --- glycoprotein duffy plays a role in inflammation --- infection. It is a member of the --- receptors and the receptor for --- plus --- .

Glycoprotein duffy expands the membrane seven times and has a --- the parasite specifically siting bind, the bind inside for --- and the major antigen demands are located in overlapping regions at the --- and terminal terminals.

(Slide)

The Duffy System is defined by three common alleles, Fya and Fyb gene that --- co-antigens, Fya and Fyb. And FYO are --- silent. That is the major allele in Africans and, of course, rarely in other populations.

These alleles give rise to four phenotypes, Fya+b-, Fya-b+, Fya+b+ and Fya-b-. Fya and Fyb polymorphism is associated with --- at nucleotide at 125 that encodes and expressed in Fyb --- Fya at terminal --- residue of the major transcript. This polymorphism is located at the first --- domain.

(Slide)

Duffy phenotype is associated with three genetic backgrounds. Fya-b- associated with Fyb-33 is the major phenotyping in Africans and it appears to be protective against malaria vivax. A mutation in the promoter region in this situation abolishes expression of glycoprotein duffy ---but not in --- cells.

In Caucasians we have two different genetic backgrounds, one due to silent alleles and there are other related to the Fyb weak allele or the FYX.

Duffy phenotype due to Fyb-33 is associated with --- in the GATA box that prevents transcription of the duffy gene in the red blood cells, but not in the other tissues. In Fyb negative and b negative persons of the

African descent the duffy gene encodes Fyb, also Fya-b- --- Fya was found in individuals of Papa New Guinea and in malaria patients from the Amazon region in Brazil.

I would like to call the attention that people with this mutation, this GATA mutation, --- express the duffy gene in the red blood cells, but they express no other tissues and they --- Fyb positive red blood cells without risk of alloimmunization.

(Slide)

There is another Fya-b- phenotype that results from silent alleles, FYAO and FYBO. These silent alleles are generated by four different mechanisms associated --- caused by either SNPs or deletion in exon 2.

(Slide)

Fya-b- phenotyping also identified with Caucasians can be associated with the Fyx or the Fyx phenotype. This phenotype is a result of two --- mutations in exon 2 of their SNPs from --- at nucleotide 265 and from --- at nucleotide 298.

(Slide)

The change in --- 8-9 and allo into --- at amino acid 100; residue 100. But the --- mutation responsible for the Fyx phenotyping 265 --- . The change --- 100 is shown not to affect the expression of cells surfaced --- protein. However, this change --- residue 8-9 show --- the translation and/or the stability of the red blood cell membrane; of the duffy in the red blood cell membrane.

This substitution leads to a reduced level of protein in the red blood cell membrane. Fyx has 1/10 level of the duffy b antigen. This explains why sometimes Fyx are not detected by the routinely used hemagglutination assays; so

that it may appear as Fya neg. Fyb neg.

In fact, in our laboratory, for example, all of the Fyx is identified by molecular testing where we feared --- Fyb negative phenotype.

(Slide)

We found in Brazilians another mutation --- from G to T related to Fyb; G to T in nucleotide 1, 4, 5 that coexists with the mutation 265 --- and 298 from G to E. This SNP caused the substitution of --- at amino acid 49. The donors who presented this mutation associated with 265 and 298 had a markedly reduced expression of Fyb, Fy3 and Fy6.

Another mutation was found in Brazilian and Asians --- at nucleotide 199 that changes --- six to seven. These mutations showed --- affect the expression.

(Slide)

Here we represent in the red blood cell membrane the different polymorphisms. We can have three combinations of Fyb. We can have only 100 --- but none 89 cystine. 89 cystine is associated with 100 --- is called Fyb2. And we have the Brazilian allele that is association of --- with 89 cystine and 100 --- that is called Fyb3 allele.

(Slide)

This is the duffy gene with the restriction site that we use to do genotyping by PCR-RFLP, and this is the PCR-RFLP that we are using to genotype the duffy and GATA mutation. This PCR-RFLP was developed by Dr. Maria Rios, and we are using our lab in Brazil.

(Slide)

We also use also --- to determine 265 mutation and the 145 mutation found in our population, and we use the --- to determine the 298 mutation.

(Slide)

Duffy genotyping has several applications, including --- transfused patients to insure a more accurate selection of compatible donor units. Fyx confirmation --- b negative samples for antibody detection and identification in red blood cell panel. The determination of single --- antigens for Fya and Fyb to identify GATA mutated duffy allele for searching and confirming rare phenotypes in --- serologic results.

And we are also using Brazil for malaria and genetic studies, and we started to use the duffy gene as a genetic marker to determine blood group specific to chimerism after genetic blood bone marrow transplantation.

(Slide)

This table shows genotyping results in Brazilian blood -- a select group of Brazilian blood donors from --- by using PCR-RFLP. Here, we you can see that we found all the combinations of the genotyping. Our population is a very diverse population. We have a very mixed genetic population, and as we can see here, we found FYB1 only. Alone. We found FYB1 and FYB-33. We found FYB-2 and we found FYB-2 with

FYB-33, showing our mixed population here. And FYB-3, the allele found in Brazil, and homozygous for GATA mutation.

(Slide)

This table shows results we saw that 36 percent of the Fyb gene were not phenotypically expressed --- GATA mutation. But in the Brazilian

population generally the prevalence of Fyb positive phenotypes is about 65 percent, and Fyb negative 35 percent.

(Slide)

This table shows results for duffy genotyping on samples from 85 sickle cell disease patients by using the --- from --- that will be presented to you by Dr. Hashmi tomorrow. In this chip we analyzed the nucleotide --- the GATA mutation and the nucleotide --- .

In these patients we did not find any 265 mutations, but we found several FYB-33 alleles. This results can give us an idea of the relevance of the molecular testing in the duffy system. --- Fya+b- phenotyping we can see that only one patient, one of 24 patients, was the true Fya. And this patient, only this patient, would require Fyb negative red cells.

Twenty-three of 24 could receive the Fya+b-, Fya-b+ or Fya+b+ red blood cells. If you take into consideration all of the Fyb negative sickle cell disease patients, we have only one in 52 who really require Fya+b- or Fyb-neg blood units. This is important because we can increase the availability of blood units to these patients.

In this population 61 percent of the FYB gene were not phenotypically expressed due to GATA mutation.

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Our studies in Brazil show us that in the Brazilian population the Fya-b- phenotype is frequent, and it is --- associated with -33 --- the GATA-1 --- thus, the molecular background of this phenotype is probably the same as in African populations. Fyb-33 should be considered in precision tests and the safe use of

Fyb+ blood and paternity cases.

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Based on this, we started in two different hospitals in Brazil, as a clinical trial, to give

Fyb-positive blood units to Fyb-negative patients who were in complicated transfusion situations and were genotyped for duffy and GATA.

(Slide)

These patients received one to 57 blood units. Three days we had 28 patients. Three patients were phenotyped as Fya+b- and genotyped as Fya/Fyb-33. They received six to 32 Fya/b+ blood units, and we had 25 patients phenotyped as Fya-b-, genotyped as Fyb-33 homozygous and they received from 1 to 57 Fyb+ blood units --- 6.5 blood units, Fyb-positive blood units transfused.

These patients did not make anti-Fyb or anti-Fy3. Knowing that the patients who carry the GATA box mutation can safely receive Fyb positive blood units --- patients in complicated transfusion situations Fyb-positive red blood cells, increasing the availability of blood to them.

(Slide)

We had also 23 patients phenotyped as Fya-b- and genotyped as Fyb-33 homozygous, and here we see the blood transfusion with units is not typed. They received from 1 to 377 blood units, and these patients always did not make anti Fyb or anti Fy3. Considering that in our population

Fyb-positive has a prevalence of 6 to 5 percent, they certainly received Fyb-positive blood units.

I know that anti Fy-3 was found in patients with GATA box mutation,

but we -- this case is -- the molecular background and the clinical significance in such cases are yet to be determined. And in our experience in Brazil we did not find anti Fy3 in our patients.

And we have, for example, a reference sample in Rio de Jenero, a reference center for sickle cell disease patients and they don't have patients with anti Fy3. So, in our experience we did not find any anti Fy3 in sickle cell disease patients or in Brazilian patients.

(Slide)

Another interesting application of the duffy phenotyping in the use of the duffy gene as a genetic marker for chimerism assessment in homologous stem cell transplant. What we are doing. We are doing microarray using the --- array from Bio Resolutions to screen patients and donors to find the mismatch.

When we find the mismatch, we select the genetic marker and follow up the patients after the transplant. Here I am presenting four patients where the selected genetic marker was the Duffy gene, and as we can see, the results show 100 percent of donor chimerism --- successful of the --- was documented and confirmed by STR and HLA results.

(Slide)

Duffy genotyping has some benefits that we are considering very important, and it can improve the quality and accuracy of the typing results and has a substantial contribution to patient care improving through transfusion management because of the high prevalence of Fyb-33 individuals, and it is important to resources preservation in using -- for the rational use of blood, preventing the unnecessary usage of --- for other antigens.

In Brazil we also identified --- alleles duffy genotyping not reported in other populations, like Fya-145 and Fya-199.

(Slide)

Duffy genotyping has the limitations of the genotype already discussed here today. Interpretation of genotype results must take into account the potential of the --- and we need also to have in mind that that presence of a particular genotype does not guarantee the expression of this antigen on the red blood cell membrane.

In duffy genotyping that you investigate the silent mutations to predict the phenotype and to select which patients could safely receive Fya/b positive blood units.

(Slide)

And, thank you, for your attention.

(Applause.)

MS. KOCHMAN: We are now going to hear from Dr. Soohee Lee, who is also from the New York Blood Center, and she is going to speak to us on Kell and the Kx proteins. This is an area where she has done quite a bit of work and published quite a bit of it. And so, she is going to teach us everything that we need to know.

The Kell and Kx Blood Group Systems

by Dr. Soohee Lee

DR. LEE: Before I start my presentation, I thank you for inviting me to participate at this workshop. I am going to describe about the Kell and Kx blood group system. In my presentation I will cover the basic information of these two

important blood groups and also the molecular basis of the antigens. Lastly I will describe some clinical importance of some of the antigens.

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The Kell Blood Group System is a major human blood group system that is highly polymorphic, expressing about 30 different alloantigens it is important in transfusion medicine after the ABO and RH systems since of the antigens are potent immunogens.

Kell antibodies can cause transfusion reactions and mismatched blood transfusions and fetal anemia in fetal maternal incompatible pregnancies.

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The Kell and Kx blood group systems are interrelated because Kell protein that carries Kell antigens and Xk protein that carries a single antigen Kx are linked on red cells through a single --- bond. In fact, they should be considered as one protein unit, but blood group ISBT rules they keep those two blood group systems separate because those are two different gene products.

Kell is 93 kDa type II membrane glycoprotein having one TMR region with a short intracellular N-terminal domain and a large extracellular C-terminal domain. Kell is an endothelin-3 converting enzyme and belongs to the M13 family of Zinc and endopeptidases. On the other hand, XK is a 50.9 kda, a putative membrane transport protein and traverses the membrane 10 times.

(Slide)

This represents a Kell/XK protein complex. The protein on the left is XK protein that has tension membrane region and five expressed loop. The second loop is large, and both -- the N-terminals and the C-terminals are

interlocated.

The protein on the right is a Kell protein that has one transmembrane region. Having N-terminal interrelated, it is a short segment and the large ectodomain. It has five glutination sites, and these regions that is the thicker lines are the residues; the portion that is mostly conserved in M13 family members.

It also has 15 cysteine residues, predicting that highly for either nature, 10 of which are --- M13 family, and this system, the non-served cysteine right after the transmembrane region is linked to the cysteine and --- extracellular loop of XK protein is linked through disulfide bank.

This biochemical relationship of a complex formation formed between Kell and XK explains the only observation that indicated that Kell/XK are related, and these are shown in two-layer phenotype. McLeod and Kelfy null phenotype.

(Slide)

And McLeod red cell blood lack KX antigen and have a very weak expression of all Kell antigens. Western blot show lack of XK and diminished amount of Kell glycoprotein. With Kell(null) red blood cells lack all Kell antigens but have enhanced the Kx antigen. However, the Western blots show diminished amount of XK protein. Probably, in the
wild-type, Kell protein partially covers Kx antigen.

(Slide)

This shows the organization of a human Kell gene. It composes of 19 exons located in chromosome, the long arm of chromosome seven at 33 and spans about 21.5 kilobase. And this HELLH, the --- sequence is the zinc binding enzyme active site, and it is coded by exon 16.

(Slide)

After the cloning of the Kell gene only the information of the -- all the layout of exons and introns, we have characterized the Kell variants; tried to find out the molecular basis of all the antigens. And here, is a list of the Kell antigens. The Kell antigens that is due to the point in nucleotide mutations.

The first four sets of antigens in these Kell 14 and 24 has an antithetical pair, high and low prevalence and the --- are independently found. And all of these are due to a single nucleotide point mutations that can be easily used in genotyping.

And here are listed the restriction enzyme changes due to the mutation, and those that has an asterisk are the research --- created by putting the mutation in the primers. But those can be easily used in PCR-RFLP analysis.

(Slide)

There are two KEL phenotypes that need to be further described, KEL1 and KEL6. KEL1 is also called "Big K" and "K1", but in older days Kell represents mainly KEL1 antigen. It is antithetical paired with KEL2, --- k or K2. It is present 90 percent in Caucasians and two percent in Blacks. And the mutation --- and nucleotide 698 the change is thrown into --- and immunize residue 193.

And this --- sequence and changing to methionine in KEL1 removes one --- residue. In KEL6, also called Jsa or K6, is antithetical paired with KEL7, Jsb or K7, and it is present. About 19.5 percent in Blacks and less than one percent in Caucasians. In the mutation at 1910 nucleotide

--- with --- .

This --- change is located between two non-conservative closely

placed cysteine residues and this antigen is much more sensitive to a reducing agent.

(Slide)

This 3-D molecular model shows --- model. It is constructed based on the --- structure of NEP Kell homolog. It has two globular domain. One is larger and placed away from the plasma membrane, and that is non-conserved in the M13 family and carries much of the Kell antigens.

This zinc is the catalytic enzyme site, and this global domain is closer to the plasma membrane and is more conserved.

(Slide)

There are two rare KEL phenotypes, the KELmod and the KELLnull phenotype, and the KELnull lacks all Kell antigens but KELmod is -- it has a weak -- - the Kell antigens, and this compilation is for KELnull genotypes.

The KELnull genotypes can be due to or caused by single --- mutation in exon or in supply site junction, and that results in --- or alternate splicing. And in the results all will not have normal Kell protein. And in one case that is marked as asterisk here, --- is nonsense mutation. It also appears in KEL1, in a sense that it is a very weak expression of Kell.

When it is paired with KELnull allele, it is identified as KELnull. But when it is homozygous for the --- substitution, then it appears as KEL1. So, in a sense it is really a KELMOD allele rather than KELnull.

(Slide)

And KELMOD is an inhibited rare red blood cell phenotype characterized by weak but detectable expression of high incident Kell antigens.

Different --- mutations cause amino acid substitutions, they may alter protein confirmation and --- Kell proteins to the cell surface. The depression of the antigen is intensified when paired with the KELnull allele. As I explained before, it could appear as a KELnull.

(Slide)

This is the compilation of a KELMOD genotype. It all is a misense mutation here. This one and this one and this one. It has very weak expression of a Kell protein and mostly is --- the transportation of the protein. And L329P change is actually KEL13, but it is called the KELMOD here.

And Kell, unlike --- antibodies, KELMOD antibodies produces specific for the immunize change.

(Slide)

I will describe about the clinical significance of Kell antibodies, especially KEL1. Anti KEL1 is the second most common antibody after Anti-D, causing complications in pregnancies and is produced mostly by incompatible blood transfusions and in some cases by previous pregnancies.

In 1986 Caine et al reported a statistical study on the KEL sensitization in pregnancy. In 127,076 pregnancies there were 127 pregnancies that had antibodies to KEL1. Thirteen of them had the KEL1 positive babies and five had serious natal complications, like hydrox and neonatal death.

(Slide)

There was another study reported by Bowman et al in 1992. 311 Manitoban women were KEL1 immunized and had 459 pregnancies, and 63 of these pregnancies ended in abortion or still births unrelated to anti KEL1. 376 were

unaffected, but 20 were affected, of which 12 did not require treatment, but four received treatment and four resulted in death.

(Slide)

The causes of fetal anemia related to antiKEL1 is different from anti-D related hemolytic of a newborn. Fetal anemia related to anti KEL antibodies is also to be due to the inhibition of erythropoiesis rather than hemolysis of red cells.

Kell glycoprotein is expressed in early erythroid progenitor cells and Anti-KEL1 related fetal anemia is caused by promoting the immune destruction of KEL1 positive cells by microphages at an early progenitor cell stage.

Therefore, anti-KEL1 titers and bilirubin levels of maternal blood are not good predictors of anemia. Fetal KEL genotyping is required.

When a mother is KEL1 negative and has a KEL1 antibody, the father should be typed to determine the potential for a KEL1 baby. If the father is KEL:1,2 phenotype, fetal genotyping should be performed to determine if further interventions are necessary. Procedures for fetal genotyping have been authenticated in early 1996.

(Slide)

Those two methods are employing PCR based RFLP analysis, another one is allele specific PCR, called another name, sequence specific PCR. The --- enzyme is created by the mutation present in KEL1 genotype. These PCR primers were designed to amplify 740 base pair PCR product and then cut with --- enzyme and the KEL1 genotype will be having two PCR -- the fragments 540 base pair and 200 base pair. But the KEL2 allele will not be cut.

(Slide)

And the other procedure, allele-specific PCR, is also based on the same PCR product, 740 base pair, but it is specifically designed only to amplify KEL1 for 540 base pair and KEL2 allele on 200 base pair product. And this was done also on amniotic fluid fetal DNA and it identified the KEL1, KEL2 heterozygous, at lane 11 and lane 18 I believe.

And also tested on --- blood cell DNA. But what is important here is the quality of the DNA as described by previous presenters. The amniotic fluid, when it is submitted to the lab, it is not a fresh one sometimes, and the quality of DNA, no matter how the technique is good, sometimes turns out not good.

So, because of the fragmentation of the genomic DNA, it is devised later that the shorter amplification of the PCR product is beneficial than a longer one. Like initially we use 740, but later people use shorter fragments.

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Now I will switch gears and describe the Kx blood group system. It is composed of a single antigen Kx. The function of XK protein is not known, but absence of XK found in the McLeod phenotype is associated with red cell acanthocytosis in late onset forms of neuromuscular abnormalities, known as Mcleod Syndrome.

McLeod phenotype is identified by serological testing by absence of Kx antigen and weak expression of all Kell antigens, but ultimate confirmation is by XK gene genotyping. The antibody to a Kx antigen is not very much available, and the quality sometimes is not good and lab to lab the result can vary.

(Slide)

The SK gene is located in X chromosome short arm at 21.1. It spins

about 50 kilo base and it is composed of three exons

(Slide)

And the genotypes for the Mcleod phenotypes vary. It ranges from gene deletion, whole or a multiple gene, to one base pair deletion to several base pair. And also, mutation can occur at splice junction or near the splice junction, and there was one base pair insertion also found.

And point mutation can occur in X coding regions and all can result in stop coding a truncated protein. But there are three missense patients that all occur in transmembrane region.

In this R22 case we transfected --- cell and proved that it can be -- protein can be translated, but it degrades and then it doesn't go to the --- in the other two cases missense mutation gene products also will be the similar case.

(Slide)

In summary, the KEL blood group system is the most important system after the ABO and Rh systems because it is highly polymorphic and some of the antigens are strong immunogens.

There are about 30 known antigens and all result from single nucleotide mutations. They change single amino acids. Genotyping for the Kell antigens is possible by utilizing PCR-RFLP or allele specific PCR methods.

Among Kell antigens KEL1 is the most immunogenic and can cause transfusion reactions in mismatched transfusions and fetal anemia in fetal maternal incompatible pregnancies.

Kell and Kx blood group systems are interrelated because the Kell protein that carries Kell antigens and the XK protein that carries a single antigen Kx

are covalently linked on red cells through a single disulfide bond.

By serology absence of the Kx antigen and weakened expression of Kell antigens identify the McLeod phenotype that manifests red cell acanthocytosis and late onset forms of neuromuscular abnormalities, known as the McLeod Syndrome.

McLeod genotypes result from various gene mutations. Gene characterization requires sequencing the PCR products of the exons and the exon-intron junctions of the XK gene. Thank you for your attention.

(Applause.)

MS. KOCHMAN: I have us scheduled for another half an hour break right now, but I don't want you asking your questions outside out in the hall. We need some really active, lively discussion after the break in here. As I said at the beginning, I am afraid there are a lot more questions than there are answers, but I need to know what all of those questions are. So, if you could all be back by 3:30. Thank you.

(Whereupon, a brief recess was taken.)

Panel Discussion

MS. KOCHMAN: I hope too many people haven't opted for an early end to the day, because I am really looking forward to some interesting and lively questions here.

As I said, if FDA is going to move forward with this, we have to try and anticipate some of the issues, and the sooner we can start thinking about them, the sooner we can get answers and the sooner things can get to market.

I will ask the first question, because when I came to FDA in 1990 there were a few monoclonal antisera on the market. It was big, hot stuff. And we thought that monoclonal antibodies were going to be the answer to all of our problems, and they weren't.

In fact, they caused more problems for us and I am keenly aware of many of those problems because I have gotten involved with some firsthand. So, one of the things that is nagging at me is how can we anticipate or can we avoid some of those unexpected problems that we had with monoclonals coming up? Anybody?

DR. WESTHOFF: Sheryl, if you would clarify for us. Many of us aren't aware of what that all involved. For example, are there monoclonals out there that aren't licensed that could be looked at to maybe make in a blend or reagent? Now that we can match a molecular or a reason for a discrepancy, are there other monoclonals that could be looked at that could possibly be on the market in the U.S.?

MS. KOCHMAN: Absolutely. Absolutely. I think it would be easier for us -- some of the problems -- I think some of you have heard me talk about the problem with the ES4 cell line and its wonderful ability to detect acquired B's. We didn't think that was necessarily a bad thing because it wasn't a wrong answer.

But what we ended up finding out was that it was doing something far better than the polyclonals ever did and was creating headaches for us. So, that product is no longer used in the United States for the manufacture of anti-B reagent.

I think we knew there would not be a perfect monoclonal anti-D, but I

don't think we knew that we would have -- we certainly didn't know about the problems with Crawford.

DR. WESTHOFF: But if you were bringing monoclonals to the market now where we have the ability to well characterize cells and know the difference between the cells, and actually, to characterize the reactivity of monoclonals, now I think it would be a different ball game and you might be able to find the perfect reagent.

MS. KOCHMAN: I think you are right.

DR. REID: And we certainly could use the molecular knowledge to create the antibodies that are needed. And I would like to get the audience going here a bit and get some input from them. I see Tony from Ortho.

Is there anything on the horizon that commercial companies are going to select, as Connie has suggested, monoclonal anti-D's so that they would be suitable say for the donor testing and another file that is suitable for the patient testing?

MR. : Well, I'd like to address two things here. First of all, the concern about monoclonals coming to market when there are available clones to select from I think there certainly are issues that relate to the tremendous amount of work and validation and fees associated with bringing a new product to market. And each time you do a new clone --

DR. REID: Who gets the fees, the FDA?

MR. : Yes.

MS. KOCHMAN: They come to FDA, but trust me, my staff and I never see them.

DR. WESTHOFF: But they are purely proportional to the effort. Right?

MS. KOCHMAN: They are supposed to be.

MR. : The other is issue concerning looking at other clones to address the needs from a donor based testing versus a patient based testing. Obviously at Ortho we are evaluating those types of alternatives to try to address the need specific to the patient population versus the donors.

Again, changing the clone goes back to making a brand new submission and going through all of that work and the fees to do that.

DR. GARRATTY: Garratty, Southern California. I would agree with Sheryl. I think we are certainly going to have some problems at blood centers for instance, just as you say, like we did when we started using monoclonals where all of a sudden we had a group of people who had been called group "O" for years and now we are typing as group "A", and it caused a lot of internal problems in decision making at that time.

And I would like to come back, and I know I keep asking this question of some of you. But the group that you talk about, they have a D gene and it is not expressed on the membrane. We are going to start picking up, presumably then, people who have been D negative for years and are now what might be called D positive, these sort of people.

It is much more important to the decision making about what importance do they have in terms of being immunogenic. I guess, from looking at your little cartoons, that we are seeing some of these now producing antibodies.

DR. REID: I think they potentially could make antibodies, but we

have been transfusing those units for 50 years. It hasn't been a major problem. I think Connie made the point that -- do we want to prevent every single anti-D being produced or do we want to target certain populations? You know, there are a lot of decisions to be made. I'm not going to give the answers.

DR. GARRATTY: No. I'm just interested in what you think about it. For instance, how do they make antibodies? Are they making antibodies because the epitope gets recognized in the atheleal system at the end of their life when they get broken down?

DR. WESTHOFF: George, I need a clarification. The ones we had been talking about were weak D's and DELs that type as D negative, but do have protein in the membrane. I don't think any of us wanted to us that if there is not protein in the membrane that we are going to have those be immunogenic red cells.

In fact, there is a well known biological phenomenon called MRNADK, which proteins that have a stop code in them or are misspliced or missense proteins are automatically degraded, oftentimes before they leave the nucleus. So, not protein has ever been found in any other system when there are those kinds of mutations.

So, in activated genes I think there is no evidence that there is any protein made. So, if that is what you are referring to, I think that is not a problem. Or, did I misunderstand?

DR. GARRATTY: I don't know if you did or not. I will have to go back and look at your cartoons and rephrase the question to you later.

DR. DENOMME: I just wanted to make a couple of comments at the

start. Monoclonal antibodies I think have done immunohematology a great justice. There is a little cost and we are seeing. It seems like a lot, but if we think back before there were monoclonals, it wasn't all that easy.

If you look at the chart that Connie had shown about Crawford -- I had a very good molecular biologist in immunology who said that monoclonals can be very good; a poor man's sequencer, and I think that is what we are seeing here.

We are seeing some things that we learned from -- that teach us. Without the monoclonals, we may still be ignorant. So, having said that, the cost -- we have to be quick on our feet. It is not a perfect world, but by far monoclonals have done a great suggest to immunohematology.

On the issue of weak D's within donors, I wrestled with this a number of times. I don't think there are very good studies that have systematically looked at the recipients who receive very weak D's, like a weak D type 2
--- r prime or some of the very low copy numbers DEL. I think that needs a very systematic and well thought out evaluation of whether these people are, in fact, immunized.

This isn't easy stuff to do. The fact that we haven't seen it -- many people who are transfused are elderly and you don't see them again. People who get eight units have the 50 percent chance they won't be around in a year. So really, I think a systematic review in donor populations where you find weak D's that are not serologically positive, we really do need to be very careful.

MS. LOMAS-FRANCIS: Just anecdotally, when RO HAR was supposed to identify -- oh, gosh. Back in the '70s or whenever that was. And found to be particularly prevalent in one part of Germany, there was a look back

because I think seven of the Rh negative donors in one center were found to be RO HAR and none of them appear to have stimulated the production of anti-D.

I think all of these rare variants that have minuscule amounts of an antigen expressed on them -- just again, anecdotally, they cannot be that significant.

There is always going to be one case where you have a woman of child bearing age who is stimulated or something like that. But if we look at all of the work that has been done over the last 30 or 40 years, that in itself has shown that these things cannot be that significant, because we are really not coming up with a huge number of antibodies being produced without a known stimulus, because whenever one is found, then basically it is a paper in literature.

Unless, of course, this happens to be in patients who are responders and it is more difficult to work out. So maybe we are losing some of those. But it cannot be quite as large a problem as we are building it up to, would be my thought.

DR. REID: Going back specifically to your question, monoclonal antibodies have taught us stuff. I don't consider them problems. They have different characteristics from polyclonal and they have different characteristics between certain monoclonals. As long as we know what those characteristics are, we can make an intelligent decision on how we want to test them or which ones we want to use.

But I think molecular is going to do the same thing. We have got to know what we are testing to test it, but I imagine it is going to create just as many, if not more, issues for you.

MR. ALLAN: Could I make a comment to defend monoclonals? John Allan, from Alba Bioscience. I don't think we see the same problems in Europe regarding monoclonal cell lanes as you seem to have here in the states, because I think there are a number or more monoclonal cell lanes available and particularly aimed at patient testing and donor testing.

I would just like to comment that we certainly are working with the FDA to try and bring some new clones to the U.S. market, and hopefully, these will help resolve a lot of the discrepant results that people are currently finding.

MS. : I am going to ask a question that I hope people don't think this is my social position on this issue. But a number of the presenters this afternoon talked about it would be beneficial to have the father tested. We were talking about KEL and Duffy and Kidd systems.

We are in an era today where we often don't know much about the father, and I'm particularly thinking about sperm banks, egg banks, et cetera. So, has there been any discussion around how we can better qualify these donors at the time of donation so that there are fewer issues during the gestation period?

MS. LOMAS-FRANCIS: This really isn't an answer, but I do know that we have been contacted by a sperm bank at one point to ask information about DNA analysis and things like that. So, you would think that the sperm banks and the like would be aware of these sort of issues and would, you know, start doing that testing themselves.

But if that is not the case, it may be difficult for us to do so. So then it almost comes back to doing fetal DNA analysis in the mother's plasma.

DR. DENOMME: We have a working relationship with the in vitro

fertilization group. We will test sperm. We have a procedure for genomic DNA isolation and a few times have insured that the sperm donor is K negative. So, we do that presently.

MS. : I actually was going to ask if there was a method for actually testing the material.

DR. DENOMME: Yes. And it is straightforward.

DR. WESTHOFF: On the east coast there is an IBF clinic that we do testing for. These are moms that have anti-D that are using in vitro fertilization. Two of the cases were mothers who wanted to use the same sperm donor for child number two, and they wanted to determine if he was a homozygote or heterozygote.

So, if he was a heterozygote, they were going to use him as a sperm donor. If he was a homozygote, they weren't; so that they would have a chance of getting a negative fetus and they were going to also type the single cell embryo and implant only the negative. So, the IBF folks are right on top of this.

DR. FLEGEL: Flegel, Ulm. I have a question for Dr. Marion Reid. You suggested that if one wants to get rid of these DEL donors one could simply use the C and E donors as D positive. That is the serology approach, which was abundant 10 years ago in Germany, and I think we should not introduce that anymore or again because 99 percent of those C and E donors are actually D negative. So, they can be used for D negative blood units.

And my question is why shouldn't one apply this easy solution at the molecular level to resolving or to fishing out these one percent D positive? Connie, if you want or respond.

DR. REID: I just say it is another approach. It is not like molecular is here and we have got to use it. There is still -- serology has value and would detect the majority of the problem cases.

DR. WESTHOFF: I may have given the mis-impression. What I was trying to convey was that this would be an initial screening for the ones that you would then go on and molecular test; so that if you had a D negative unit that was C or E positive, those are the ones you would be molecular screening rather than all of the D negative donors. So, I think I contributed to that misconception.

DR. FLEGEL: Okay. Then I would have the next question. How do you want to tackle the problem with the D+/- chimera? Because they will occur by statistical reason amongst c and e D negative donor populations.

DR. WESTHOFF: Well, unfortunately, like the rest of you, it probably doesn't become an issue until it happens to me. I mean unfortunately the chimera issue is a difficult one because I think it is pretty unclear how frequent. You have some data and I respect that.

The chimera issue, yes, you are right. Those would be transfused as D negative units. That is why I think a policy of following up on individuals who are D negative and you know you have given them D negative blood and they have an anti-D, let's take a look at those. We need the data.

DR. RIOS: Mario Rios, from CBER. It is a highly complex gene. I actually go to everybody in the RH field. How can we really define a D negative? Because from the complexity that is there, ranging from the absence of the gene, the pseudo gene and all of those polymorphic situations that are out there through the -- you know, the D epitopes. How can one really define a D negative right

now?

MS. LOMAS-FRANCIS: Good question. I think we can say that if we show that there is a deleted D gene, then that would be a D negative. If there is a pseudo D, that would be a D negative. Some of it is going to be a combination of DNA analysis and serological analysis to see if we have an insert of D into a gene that actually encodes a product.

And I'm really hesitating on this because it is getting more and more difficult on one level, but it is not so difficult on another, because we are now seeing these genes that have a tiny bit of D and CE, and we are able to detect those. But again, these are the outliers.

If there is a D deletion or a pseudo gene or a silencing, those are D negative and those come up as such.

DR. RIOS: Are those epitopes of D expressed in C immunogenic? Is it known?

DR. WESTHOFF: I don't think it is well known. Does anybody have any anti-D's? I hope I conveyed this; that the RO HARs and the Crawfords are problems on the patient side I think. I don't know of any Crawford donor that has been shown to stimulate -- I guess it would be anti-Crawford. Or D-HARS in the literature that have stimulated anti-D.

John or some others may know those. So, I think we can have a very straightforward algorithm to determine the D negative unit. I think it will be pretty straightforward.

DR. RIOS: So, if I interpret your answer correctly, yes, you do know that there is D and DC from the patient side? But from the donor side there is no

data showing that if a donor has that mosaic or that mixture of gene, it is immunogenic? There is no data out there to support that. Okay. Thanks.

MS. KOCHMAN: Dr. Flegel and Dr. Avent, would you want to come down? I can drag up some more chairs.

(Pause.)

DR. STRONECK: Dave Stroneck, Bethesda. You mentioned earlier that you had a lot of issues with monoclonal antibodies when they came up. I think with this molecular method there is the possibility of so many probes that several years from now you won't have a lot of issues. This is really going to be a powerful platform, and it is several orders of magnitude more powerful than monoclonal antibodies.

The question I have is how do we get form where we are today to that point? From a science point of view, it is really going to be pretty straightforward. But from a regulatory point of view, you know, we have to license these at some point.

But the questions that come up is, okay, if you are going to say a test, a platform, detects ABO, how many of the 150 ways to detect an O antigen do you have to have on that platform? All of them? And then what happens when you find new ones?

And then, for example, if you have a simple kit that can test the difference between Jsa and Jsb, do you have to have something in there to make sure you don't have a KELMOD in there? Or do you use a combination of monoclonal antibody to pick up the KEL to know it is expressed so it is not KELnull and then go to Jsa and Jsb?

I really don't -- that is the other issue. I am not sure that we are ever going to get rid of serology. It might be with us even when we have very robust molecular tests.

DR. AVENT: I was going to make a comment about the monoclonal anti-D's. I think you mentioned five that were registered for use by the FDA. At the ISBT workshops that we have had since 1990 or so -- I guess there has been -- I have got about 80 or so monoclonal anti-D's in my freezer, and there are always going to be partial D's that are going to type as negative with at least one of those monoclonals.

To select one monoclonal antibody, you are going to find a partial D variant. It is always going to be negative with that. The question is how is that person, if that mistyped as D negative and they are actually a partial D, what is the likelihood of them receiving a D positive donation if they are a patient?

Of course, if they are pregnant, they are quite likely to have a D positive fetus. So I guess there is always going to be a problem with the use of monoclonal

anti-D. I think the point was made this morning that polyclonal reagents avoided that problem because you were testing for the complete D antigen, not just one D epitope.

So, there is always going to be an inherent problem with detection of all partial D's with the use of one or two monoclonal reagents.

Now, those partial D's individuals may be extremely rare, one in 30,000, but the combination of them, when you add them together, we don't know the number of partial D's that are actually out there. And I think the cataloging that

we are attempting to do at the moment -- we are making, I think, very good progress. We have a true indication of the numbers of partial D phenotypes that are out there. I think we are only partially there at the moment.

MS. LOMAS-FRANCIS: Can I just make a slight comment on your question? You said are we going to have to do some testing, for example, if you are looking at a kit for Jsa and Jsb, to determine if a null phenotype is involved?

And the answer to that is it depends where you are doing the testing, because if you are doing that testing to identify a donor, as being say Jsa or even Jsb negative, then if -- that test shows you that the JSA or the Jsb gene is present. So, if you want your donor to be Jsa negative, but it is showing Jsa positive, it doesn't actually matter from that point of view if it is a null phenotype, because you are going to reject that donor because it doesn't fit the criteria that you are looking for.

So, with regard to the donor, it really doesn't matter. With regard to the patient, in certain situations that can be a complicated scenario. One would hope that we all of the information we are gathering we would know when we would need to do additional serological testing or even additional DNA testing to look for silencing mutations and the like.

So, it comes back again to gathering more data and looking for the silencing mutations and sequencing.

DR. NANCE: Nance, Philadelphia, with the Red Cross. It seems to me that this is really not a whole lot different than the Olympus question, in that there are limitations to the technology. And if we recognize them upon licensing and we do our due diligence, it should be very much. We miss weak D's all of the

time on the Olympus.

DR. WESTHOFF: I would just like to paraphrase again what Christine said. The take home message here is that false positives in genotyping are not anticipated to be a problem in the donor side of the equation, because then you disregard that unit. It doesn't appear to be negative, and you are usually screening for antigen negative units. On the patient side it is a different issue.

DR. SIEGEL: I have a question. Don Siegel, University of Pennsylvania. I would like to get comments on what people envision 20 or 30 years from now in terms of what a blood bank for transfusion service in the hospital does. Because at one extreme, if all donor blood were -- its complete phenotype were known based on genotyping on some incredibly large chip and every patient had the same thing done to them, whether it was when they were born or when they got their driver's license or whatever -- I mean in theory no one would get blood that would have an antigen on it even if they had an antibody. So there wouldn't be any reason you would have to do antibody screens or antibody identifications.

I mean, theoretically all a blood bank would really be is just a depository of bags and you would just sort of run things in and out. There would be actually no testing at all.

On the other extreme -- or not the extreme. Then another variation could be where you confirm things with antibodies, or not, or you just don't do screens or panels. You just do crossmatches on everything just as a final check that all of the genotyping was right.

So, I just wonder. Maybe this is a question for tomorrow afternoon,

but I am just wondering at this point what do people envision will be the outcome of all of this?

DR. AVENT: I think that all of our genomes will be completely sequenced; I think in 20 or 30 years time. I think we won't be messing around with testing platforms. I think the genomes will be completely defined.

So I think the possibility that you are --- to a particular disease is if you have got some metabolic disorders. Your HLA type will be defined in advance. But there will always be, and I think there will always be, human error. So there does need to be a fail safe mechanism, and the antibody detection certainly still needs to be done for blood transfusion, if we are doing blood transfusion in 20 or 30 years.

DR. SIEGEL: So then the question is when do you do the antibody? Is it to check the phenotype of that rare unit that you found by screening using genotyping methods? Or is it -- when would you envision in 30 years you would use? What would you want to check on? What kind of errors would you want to look for, just ABO errors?

DR. WESTHOFF: The computer takes care of the blood bank inventory. It takes care of selecting the unit for the patient, it takes care of sending them to the floor and your bedside test confirms the ABO type and the unit is transfused.

DR. SIEGEL: So, no more gel cards or --

DR. WESTHOFF: You said 30 years. Okay. Thirty to 50 years.

DR. RIOS: Am I being naive here? We are forgetting the availability of donations itself. I think some of the problems that we have now is not that we

don't have the ability to match even with the serological methods, a complete phenotype. Sometimes we don't have the availability of that donation.

If we will have everybody donate a unit every year, every single human being, then we may be able to match everybody perfectly. Again, I may be naive. But I don't think in 10 or 15 years we are going to have enough inventory to assure that everybody is going to be able to get a complete phenotype match or genotype match unit.

DR. FLEGEL: We may disagree at the moment whether it is cost efficient to do this, to make it compatible on a 100 percent level. But I think we do not disagree that if it would be possible, then the best blood would be 100 percent compatible blood.

And in the long-term, 20 or 30 years, this could become technically feasible, cost efficient and possible to a large extent. We should go for that goal.

DR. WESTHOFF: And we should also go for changing donor recruitment or thinking about ways to change donor recruitment. It certainly doesn't work necessarily all that well right now. I mean, it is not a system that I can say in the U.S. necessarily works well.

So, being innovative about donor recruitment, whether it is a professional donor. Kind of the sky is the limit to start thinking about this.

MS. KOWALSKI: Mary Kowalski, Kansas City. I may be showing my lack of immunologic knowledge here, but when we are talking about finding match donors, we already know for some at least platelet antigens and maybe some red cell antigens that you have to have a matched HLA type 2 gene in order to even produce antibody. So I'm wondering if that isn't going to be part of that picture; that

you are only going to provide antigen negative blood for the patient who is capable of making antibodies.

And then Dolores' point, that it is going to be hard to find the matched unit. You would have many fewer patients that you would have to match for.

That may be an oversimplification, but I wondered if we thought about that.

DR. DENOMME: Very good, Mary. If you think about a unit or a platelet or a unit of blood, it is a drug. And if you look into the drug industry, they are very interested in how people metabolize a drug. Some people metabolize a drug differently.

I think within our industry we will be able to identify patients who are set up to have adverse immunological transfusion reactions and predict that before it happens. It may take 20 or 30 years, but I think it will be done.

And possibly what would happen is that based on that information a full match wouldn't necessarily be all that cost efficient, which I think has to be addressed as we go along. Or it may not be possible.

So, if you get your driver's license and you realize that you have certain metabolic -- a potential for a metabolic disorder or a potential for an adverse immunologic transfusion reaction in a certain way, certain blood group systems, then that information is retained and it is dealt with effectively at the time you need a transfusion, be it 30 or 40 or 50 years later.

DR. REID: Consistent with that is the fact that in all of the transfusions about two percent of patients make antibodies.

MS. : I am trying to anticipate the kind of information we are

going to have to put in our RHIG brochure. So I am going to move to the Rogam issue, RH immune globulin.

Dr. Flegel put together a very nice chart in one of his slides that suggests what the RHIG therapy should be for the basic weak D classifications. So what I am trying to do here is anticipate -- here is a specific question just to make sure I am understanding this table appropriately.

If the mother is weak D type 11, we classify then that particular woman as being RH negative and should be considered for RHIG therapy. Then match tot eh type of the baby. If the baby is weak D type 1, coupled with the mother who is weak D type 11, would the assumption be that that is a mother who definitely qualifies for rogam even based on the baby's weak D status? And it gets more complicated, but for example.

DR. FLEGEL: Yes. Yes. So, weak D type 1 baby in a D negative mother would qualify for anti-D prophylaxis and this should theoretically also apply in the case that the mother is weak D type 11. But the table was designed for D positive babies in weak D type 11 mothers and there the anti-D prophylaxis is required. Yes. Whether it is helpful is not established.

But in lack of other evidence we should continue with the current practice, and this is applying the anti-D prophylaxis in weak D type 11, weak D type 15, 4.2 and so on in mothers.

MS. PATTISON: So, do we anticipate the clinicians will be using -- oh, Paula Pattison, Ortho-Clinical Diagnostics. I am wondering if clinicians are beginning to deal with this level of detail when they are writing the script for RHIG.

DR. FLEGEL: This is an issue for a transfusion medicine specialist.

The clinicians cannot handle. This needs to be done by transfusion medicine people. We do the typing, we give the advice and, at least in Germany so far, the -- internal medicine physicians are following this advice. That is the way I think it should be because this is our specialty and we should handle -- we have to make the decisions and live with the consequences.

DR. DENOMME: And I was just going to add that some of the obstetrical guidelines for obstetricians are dated, and so that type of information up to date needs to be provided by the transfusion medicine community.

DR. WESTHOFF: The practice in the U.S., of course, differs than the social medicine situations. Our approach is to not give clinical advice, but we do, in our reports, site the literature.

For example, we have a mom who is weak D type 2. We site the literature; that to date it has not been shown to be a problem causing HDN or immunizing the mother, and we give the references to the literature. And os, the clinical decisions are made by the clinician in the U.S.

DR. CASTIHLO: The same situation in Brazil as Connie said.

MR. : I was just going to throw out do we know if RHGAN would work in those settings anyway? Let's say you need a certain amount of anti-D to coat the fetal cells, and most of it is sopped up by the maternal cells who have some epitopes that would bind. Would there be enough of the drug coding the fetal cells to clear it if that is the mechanism by which RHGAN works or to do whatever it is that RHGAN needs to do?

DR. AVENT: Anna Salla Banker and Marcela Contreras in the UK did some work on that with D6 and proved that the anti-D that was administered was

not sufficient to completely coat the maternal cells. Of course, if you get a partial D with very high D site numbers, like D5, that might not be the case.

DR. WESTHOFF: It has not been shown that you need to code all the sites. Certainly, since it is a B cell mediated situation, it has not been shown not to work. So, that is the logic. Right? And it has not been shown to be detrimental.

MS. LOMAS-FRANCIS: Continuing on the anti-natal situation, maybe it can be expanded to all patients. There is work underway to see if the immune response can be modulated.

For example, Stan O'Baniack in Scotland is working to see if certain peptides can be used to inhibit the response to anti-D within the D negative system. And so, as more and more is known about the immune response, we can start looking at other antigens to see if there is some way of inhibiting the immune response.

I think in the future whatever we do is going to be very much a multifaceted approach. There is going to be no one approach or a system that is going to answer all of our questions, unless we go to our oxygen carrying substitutes.

MS. : This is Nina --- from Red Cross. When you start thinking about how people would start treating like weak D's or partial D's or whatever, it is always based on like is there a case that was reported in a weak D, if they made anti-D. And so, when do you decide that it is significant, that that weak D could make anti-D?

The idea is you want people out of the -- like the transfusion

medicine, to know how to classify a weak D. Do you then say that this weak D can now make anti-D? And then if you, all of a sudden, get one reported where a weak D that was never reported to make anti-D now does, then it changes that recommendation. Does that --

DR. FLEGEL: The argument went the other way around. We assumed that weak D needs anti-D and then it was proven that quite a number of them, in particular, luckily, the prevalent ones don't need anti-D. That is the kind of argument.

The less frequent weak D types we simply don't know whether or not they need this anti-D, and in this case we say let's keep on with our current practice of administering the anti-D. And then there are basically three exceptions at the moment, 4.2, 15 and 11, where it is proven that there is an allo anti-D production. This is the exception; that the vast majority of the less frequent weak D types it is simply assumed that they may produce allo anti-D, although we don't have any evidence for it.

And then, we keep continuing with our practice of administering the anti-D.

DR. REID: I don't know if it answers your question, but in my mind once a certain type of partial D or weak D has made anti-D, then you say anybody of that weak type or partial D has the potential to make anti-D.

MS. : I guess that is what I was thinking more; is once you find that it has been reported to make an anti-D -- you know, say in one of the weak D types for hundreds and hundreds of people it has never made anti-D and you find that one case, now do all these weak D's then in the future you have to then

say, you know, they can make that anti-D?

Would you need a lot of people then, especially in one of the common weak D's, one, two or threes. Do you need a few people to make anti-D before you would really want to say it?

DR. DENOMME: There may be an argument for a consensus panel to get to those conclusions. But while we are here, and we talked a little bit about R-HAR, we can use the same kinds of arguments without doing experiments or looking into it further. We know that some D6's make anti-D. They are missing exons 4 and 5. RO-HAR, in an RH negative, has the D exon 5 and we know that RH negative people must have D cell clones that can produce antibodies that bind to that.

So therefore, we don't need to study RO-HAR. If a donor is RO-HAR positive, they should be considered. That unit should not go to an RH neg. If you were to happen to find it in a baby, that mother should get RH immune globulin.

We don't really need to do any other experimentation because what we know about D6, category D6's.

DR. RIOS: Maria Rios, from FDA again. You just touched on a point, Greg, that I thought was very important, because we are talking about taking care of people, patient care, and we are discussing at the same time how to type blood from a donor that will be a --- patient that needs badly that blood to survive.

So maybe we have to find a way of recommending that certain patients would be fully typed or genotyped or phenotyped up front and then come to try to match. First of all, I don't believe the whole genome, the human genome, is already sequenced. But the polymorphism that we have there is so tremendous

that I doubt that we are going to control everything.

So maybe a D1 that is getting the care needs to be fully genotyped, if you will, or phenotyped together. I would like to hear the presenters' discussion about that.

DR. DENOMME: Well, some of the things we can do are, more or less, a shotgun approach. We can solve problems using monoclonal antibodies. I have no problem with that at all. I wouldn't want to use a monoclonal that identifies a D6 type one on immediate spin in a transfusion institution. I would want to use it if I were in a donor setting.

If a monoclonal recognizes RO-HAR, it is a monoclonal that recognizes it. It is very, very useful. It is a poor man's sequencer and we can get a lot of mileage out of that.

And as we learn these things, we modify our behavior. We have been doing it since 1901. So I don't see why it would change. I think that there is enough human variation that we will continue to learn, but by and large, we can make some very general statements and we have made them in the past.

D6 type 1, type 2, type 3 are RH negative if they are pregnant, and we can continue to do that either with either tool or even a complex tool that is part antibody and part DNA. I think they are all very, very useful tools.

So, what needs to be done is something like that RO-HAR was a very good argument; a very good example. I don't think much more needs to be done. We can make positive statements. I have asked this before, like you have asked. Is an RO-HAR -- an RH negative RO-HAR they considered RH negative or RH positive?

And it is clear from what we know immunologically that they should be considered RH positive. It is done.

DR. RIOS: But there are some cases like what Christine presented about Kidd. From her presentation it was very clear that the difficult of serologically determined Kidd and how to treat the patient that makes Kidd or a Kidd negative and so on, it is really very difficult because of the monoclonals.

In this situation maybe it is appropriate and rightfully indicated that the patient that is receiving the unit be really well characterized, as well as the blood to be transfused.

DR. DENOMME: Sure.

MS. LOMAS-FRANCIS: I think, with due respect, Maria, we can almost put ABO and RH aside because they are so complex because of the number of alleles and because of silencing and things like that.

Within Kidd, yes, there are silencing alleles, but to my mind at least Kidd is a different situation. We have a basic polymorphism Jka and Jkb, and the difficulty is that there are times that we can't use antibodies to type the patient's cells, for whatever reason; if they are multi transfused or whatever.

And so, we can establish the patient's phenotype using DNA analysis, and that is very important in some clinical situations. For example, when you are trying to figure out if it is an auto antibody or an allo antibody and how would you then deal with that patient.

And then the other situation within Kidd is because the antibodies will so often fade in vivo and you get this animistic response, once a patient has been transfused or maybe even from the first transfusion on, should we match the Kidd

type? So then we are back to typing more donors.

But on one level that is a much easier question to answer than all of the things that we have been talking about for RH or for D.

DR. CASTIHLO: We tried to match some sickle cell disease patients in Brazil using the --- sheet from Bioarrays, because we are having -- they were being alloimmunised for different antigens. And what we saw that -- we found matched blood for different antigens for these patients.

We have --- problem with RH because we need to better characterize the RH, mainly for sickle cell disease patients, because they have --- but for the other antigens we are doing that and we are having very good results.

MS. NANCE: I wanted to go back to the D or anti-D monoclonals again. I apologize for not being on the forefront hospital information, but are we on any path to have a patient anti-D that would characterize these D variants as being negative in the U.S.?

MS. KOCHMAN: We had a manufacturer who had a reagent that was labeled specifically for donor testing and a corresponding reagent that could be used in either donors or patients, and it is my understanding that the scheme didn't go over well. So, that donor anti-D is no longer available.

I don't think anyone has embraced some of the concepts that have been used in Europe in terms of treating the donors differently than the patients. I mean, as far as a manufacturer. I mean, I think the users are doing that to the extent they can, but that the manufacturers have not come along with that.

And it could be something as simple as educating the users about how to use them. We put a lot of effort into getting the package insert right, only to

hear that people don't read the package insert. We at FDA are willing to deal with anything a manufacturer wants to come to us with, but I think that they also realize they have to deal with the customer complaints, they have to deal with the people calling and saying there is an inspector here telling me I am using the reagent incorrectly and I thought I was following the package insert, but whatever.

I have concerns about the different levels of competency here in the United States, and clearly there are people who I would not want attempting to do genotyping at this point in time. And I am wondering if it will be universal in the U.S. or if it will be in certain regional centers.

That is one of the questions of where we are going to go with this. Is this something that will be in every transfusion service? Is it something that will be in every blood center? I certainly don't know the answers to that.

(Pause.)

DR. WESTHOFF: Seeing that the panel doesn't either, certainly at this point it is appropriate that the testing is done in reference lab settings. You know, this cost burden of doing it is appropriately in reference lab settings.

Now, what you see for the future, as far as the technology expands, certainly in Don's 30 to 50 year profile it certainly would be different, because it certainly would grow, et cetera. But I think most peoples' vision, and maybe in Europe too, is that there won't be one on every block, because there doesn't need to be in the era of transportation and things like that.

DR. MOULDS: Joanne Moulds, Shreveport. I just want to make the comment that this has the potential of really kind of changing the blood banking community as we know it today. There are certain centers, Peugeot Sound is one

that comes to mind, where there is centralized crossmatching. Everything is done by the blood center.

And the day may come when since it is the major blood centers that are doing the genotyping and providing the blood, that there will be no need for a hospital transfusion service. Everything will come from the blood center. So, it was just a thought.

DR. AVENT: The National Blood Centers in the U.K. is going along those line. The National Blood Service is going to be running the hospital blood banks. That is what is going on in my country at least.

DR. FLEGEL: The economies of scale and there is --- quite a bit of centralization to get this thing going. However, the genotyping will contribute significantly to the personalization of any medication, and we in transfusion medicine should not lack behind that. This is an opportunity for transfusion medicine, and we should take that.

DR. WESTHOFF: I have a question to the genotyping platforms and the sensitivity. Is it possible that these are sensitive enough to type for maternal plasma?

DR. AVENT: I think some of the genotyping platforms certainly are. Yes. I think Ellen van der Schoot has tried it, and it does work.

DR. WESTHOFF: And the other question is I would like to hear some discussion about the potential of using fetal typing from maternal plasma to eliminate the administration of anti partial immune globulin and what the vision in Europe is about the potential of that going forward.

DR. AVENT: Well, in the framework six program and the E-funded

program, which is very much looking at that, Ellen van der Schoot is the person and Jeff Daniels as well. It is very much going along the lines of genotyping all --- from rhesus negative mother at about 10 to 15 pregnancy to eliminate the use of prophylactic anti-D and to --- prophylactic anti-D, which is done at 28 and 32 weeks.

So that program is actually going ahead quite in force, and I think Ellen and the Sangrin diagnostics in the Netherlands will be doing that very soon. In the next year or two. Genotype all D negative donors.

DR. WESTHOFF: And I assume that they have determined that it is cost effective, the cost of genotyping offsets the cost of the RH immune globulin?

DR. AVENT: I think Ellen has done some --- on it and with the University of Amsterdam Medical Center, and they have come to the conclusion that it is economically viable. But, of course, in Europe and certainly in the United Kingdom we have got an issue with blood products, the use of blood products in vulnerable patient groups, et cetera.

So the elimination of the use of a human derived blood product is one of the major drivers. But there are other converse studies as well which suggest that the economics is marginally only in favor. So the economics argument is still out I guess.

MS. LOMAS-FRANCIS: I think there is more impetus on what I heard at ISBT for eliminating the use of RHIG whenever possible because, for example, there has been contamination of the batch with hepatitis C virus, and that was a huge issue in Europe. So, everyone is very sensitized to that.

DR. WESTHOFF: Would anyone care to comment about the liberal

use of RH immune globulin in the U.S. and the potential for scaling any of that back?

DR. BELLISSIMO: Dan Bellissimo, from the Blood Center of Wisconsin. I will make a comment based on some conversations I have had with OBs regarding this. Their main comment is that the health care system in the United States and the legal system in the United States most likely would not be amenable to using this kind of screening to withhold RH -- to withhold anti-D during pregnancy because of the legal ramifications of getting someone sensitized when there was a treatment for it would be too large. But that was just their opinion.

DR. WESTHOFF: Any discussion about letting the patient decide?

DR. BELLISSIMO: I think something like that -- you know, we deal with all kinds of problems like this in genetic disorders and how well patients understand risk and I think this is a very complicated risk argument. I think it would be a matter of whether that could be adequately displayed to a patient to make an informed choice.

DR. DENOMME: I would just like to comment. I have had the same discussions with obstetricians. They are very, very interested in a non-invasive test for fetal risk, but it is a slippery slope. I know the minute that we have that test for hemolytic disease of the fetus and newborn I will immediately get a call.

And there will be a patient who is very up to it, has been on the internet, knows what is going on in Europe, they are going to say, well, I get a choice if I'm in Europe. Why can't I have a choice in Canada or the U.S.?

And the obstetricians always tell me this. They spend an inordinate amount of time getting informed consent for RH immune globulin, and it really does

boil down to giving a blood product versus a test that may or may not be that robust. I think there is only good hope for it.

From what I see of Europe, it is very good. So I know immediately that in the pressure I have right now to get the test going for hemolytic disease it is going to push it down that slippery slope. And the reason I tell you that is because of our experience with fetal transfusion.

In 1992 there was a program started in Toronto where a mother -- when they delivered a child, a premi, had the choice of donating blood to her own infant, along with her husband. So they went through a screening process and an informed consent process. And it just so happened that a woman with anti-D hears that another woman in the next room is able to donate blood for her baby, and she would like to donate blood for the post-birth but exchange transfusion.

Well, the hospital then began their own blood donation process because pregnant women can't donate at the blood center. Immediately after that the woman wanted to donate blood during pregnancy and use it for inter uterine transfusion.

So, it is not black and white. It really is one encompassing deal, and if you can detect a fetus who is RH positive, you have to be prepared to entertain these discussions. And it is completely the opposite. Obstetricians are just dying for this. That is the way it is.

DR. SIEGEL: Just to follow up then. I would differentiate between the two. The OBs definitely want this test in the United States. I think it has great value for determining -- picking up RHD positive fetuses. I would just differentiate that between the choice of withholding anti-D versus them wanting this test.

DR. DENOMME: I agree. It is a huge problem we will have to face one way or the other. Well, no. I wouldn't call it a problem, but a dilemma. I think there is a very good reason to do it in hemolytic disease of the newborn.

In Canada the jury isn't out. It is the safest product that they know of. There has never been an incident, et cetera, et cetera. That doesn't mean it won't be a risk in the future. That doesn't mean that Canada isn't looking at reducing risk where they can, but they are looking very closely at a test that right now has to be done, I think, three times when it is negative.

There is a bunch of markers. It is a little bit complex. The cost is about the same. So really, people are waiting. And I would just have to say Europe is ahead of North America on this. Once this is settled, we will have to face this decision.

DR. AVENT: If the prenatal testing is 100 percent diagnostic accuracy to avoid the use of a human derived -- and I still reiterate it is a human derived product and there are risks that go with that.

I think that it is wrong to actually say to the mother you cannot do this. Certainly that is the European perspective on it.

MS. PATTISON: Paula Pattison. So, would we feel differently about this if we had a monoclonal solution for RHIG?

DR. AVENT: You get back to the human monoclonals. Are they good enough to clear red blood cells from the maternal circulation? I guess some of them are. I think it is probably a long way off though, the monoclonal reagents.

(Pause.)

DR. GARRATTY: Could I just ask the panel? In their response to

Dan Siegel about the 20 to 30, somebody mentioned a 50-year time frame in terms of where we are going to be, would I take it that you are all unanimous that at that time we would not have a blood substitute that will survive a lot longer than the one they are using now, which would put us completely out of business for what we are talking about?

DR. REID: Maybe we would be growing vats of stem cells or each have a transgenic pig in the backyard.

DR. DENOMME: I think there is probably a few approaches for the next 20 or 30 years and those approaches will come and go. Some will survive. Some will die. So, I don't know if monoclonal anti-D will replace RH immune globulin. I don't know if tolerogenic peptides will.

But we have these kinds of research in case they do. We all can't do fetal DNA for maternal plasma, but those that do the very good studies, we look very closely. And eventually those types of studies are mirrored for whatever reason. So, I don't have an answer.

Will you be able to donate your own red cells and they sit in your bedroom? I have no idea. But we do have a few approaches to some of these problems, and we don't know how they will turn out.

MS. LOMAS-FRANCIS: Can I throw out a question to the audience? I think probably all of the talks, one way or another, have shown you the power of DNA analysis that is out there. They have also shown you that it is a technique that is very valuable, but that is even more valuable if you interacted or if you also have serological testing.

Considering you as the end user, what are your thoughts about what

we should do? Should we start implementing and trying to produce more --- having centralized testing, as was discussed earlier? Where do we go from here? How do we move forward?

MS. FIGUEROA: Dolores Figueroa, from Blood Systems. I think that both are going to need to be used. At least for a long while. I also think that it is time that we as a community take a stand, and I know in the United States it is very difficult to establish who says what and what is done and what is convenient.

But we need to have specific rules that are dictated by or established by the people that knows the field in what needs to be done and tested. What a donor needs to be tested by or with or what a patient needs to be tested and the resource for that. And I think we haven't done a good job establishing those.

But in terms of the testing I think both methodologies are needed. For a long while they are going to run together until we establish or feel comfortable with the new one that is -- well, I don't know if you call it new one anymore, but the one that is now more prevalent. Or it will be.

DR. AVENT: I would like to make a comment. DNA testing and use of DNA techniques as a chemical reaction is very, very relatively straightforward to perform in the laboratory. The key issue is the interpretation of the data.

And transfusion medicine specialists, you have a job for a very long-term future because the data that is generated from DNA or serological reactions will always need interpretation in looking at what the clinical implications of that data is going to be.

So I think that that -- you know, you just see it as another tool in your

armory that is available. It is going to become available to you in the next few years.

MS. NANCE: Nance, Philadelphia. In response to your question, Christine, speaking as the Director of the American Rare Donor Program, I would certainly like to see us get the donors tested ahead of the patients because it could only be a nightmare for me if it is not.

And I don't know what the future of the American Rare Donor Program would be, but I presume it will probably be about the same as it is now. You know, it is just a whole different level of matching activity.

But I do know with the sickle cell matching that we do in Philadelphia we want to be ahead of the need. We want to have the supply there before the demand is happening. DR. WHITSETT: Carolyn Whitsett, Mt. Sanai, New York. One of the things that prevents hospitals from buying into new technology, at least in the United States, is the cost of paying for the new technology. So, if you expect blood centers to be able to roll out and centralize this new testing and hospitals to use it, you have to set the pricing at a level that allows the hospital to take advantage of this new service and be reimbursed appropriately by insurance companies so that they are encouraged to do the right thing.

Because if the price is too high, we are not going to be able to get patients genotyped at the blood center. Now you know how much I appreciate your work because I send samples and say, sequence this for me, there is something wrong with this type.

So, I know the value to the hospital of having this done, but the pricing has to be right or it just isn't going to happen in the United States in the way

that we need to have it happen.

MS. LOMAS-FRANCIS: So based on that the first move is to talk to the insurance companies and see about reimbursement, which is a problem anyway. So, we need to lobby.

MS. KOWALSKI: Mary Kowalski, Kansas City. I'm from an independent blood center, and so I'm probably looking at this in another way. I'm not from a big conglomerate.

But we are going to have to do this too. I mean, the amount of antisera that is out there now is very limited and we have micro methods and we screen all of the time. But just the fact that it can be mechanized, I'm running out of techs. You know, I can't find people to shake the tubes or do the marker plates or do any of that kind of stuff, and I think it is going to be the same across the whole nation.

So, I think that this gives us a technique that is going to allow mechanization, that is going to allow major kinds of screening. I don't know that I believe that we are going to genotype every patient in the future, but I do believe that donor centers are going to be using this because we are going to have to.

I also believe that there are going to be two levels. I mean, I think people will be using smaller and different kinds of technology maybe to look at a particular donor because they think they are rare, and there will be ones that you just use to do mass screening, the same way there are different serologic techniques that you use to look at special donors who have unusual serologic reactions. So, I think it is going to be a mix. I pass to my colleague in the front.

MS. : I think one part that we need here, and we are talking

about mass screening, is automation. We really need to be at the front of that because it will make it very difficult for a large blood center that wants to embark in a large project like this if the automation is not there to support because of what Mary just mentioned, the lack of people to be able to do some of this testing or the time needed for it to be done.

Also, I was thinking that we may want to -- I'm sorry. I didn't catch her name. Her approach. We may need to take the same type of approach that some of the drug companies are taking and going directly to the patient and say ask your doctor for this. It will be the same thing. Why this is not available to you.

DR. JOHN MOULDS: John Moulds, from LifeShare in Shreveport, Louisiana. The comment is doesn't the presence of BG antibodies in polyclonal agents seems to be quite trivial right now?

Second is if a blood center is typing units of blood by the DNA method on the units of blood and finds that they lack a duffy or a Kidd antigen or gene, can they label that kind of blood as negative for that factor and are they required to testify on the FDA license serological reagent for shipping across state lines?

MS. KOCHMAN: Currently blood establishments have BLAs, if they are licensed to ship blood across state lines, and with that BLA there are associated SOPs. So, if their SOPs that are on file with FDA say that they are going to do things serologically, then they have to do it serologically, whether they do the genotype first and then confirm serologically or not.

We actually are just beginning to understand the kinds of questions we need to ask about genotyping. So I would say that it is possible for a blood

establishment to ask now can I switch from my serologic method to a molecular method. Here is how I am going to do it.

One of the things that will make a difference is if it is "home brew" or if it is a kit that FDA has done a pre-market review on and allowed to proceed to market. Because if you are using something that the FDA has cleared and it has gone to market and you are using it exactly according to the directions for us, that is pretty much all you have to tell FDA.

At least that is my understanding. I don't deal with the blood center licenses, but that is my understanding; that if you are using an FDA cleared or approved test and you are using it exactly according to the directions for use, all you have to do is tell us that is what you are doing now.

And then, during an inspection, the investigators will evaluate whether you have appropriately validated it, whether you are running appropriate controls, whether you really are following the package insert. So your biggest problem at this point, I think, is that there isn't anything available in the U.S. that we have looked and allowed to go to market for anything -- for in vitro diagnostic use.

When it comes to home brew, that actually falls under CLIA more than it falls under FDA. And I will get into a little bit about home brewed tomorrow, but somebody has got to be the first I'm afraid to say.

DR. JOANN MOULDS: You just raised another issue that I wanted to throw out to the committee that I thought of this morning; was the issue of controls. I think everybody does a water control when they do DNA.

But if you are using a b chip or the microarray system where you are typing for many different genes, how do you do a positive and negative control for

each of those genes? Or do you?

MS. KOCHMAN: Again, this is one of the questions we have just begun to realize that we need to deal with. The CBER statisticians are already looking at microarrays. Not specifically for these analytes, but they are aware that microarrays are coming. They are trying to be prepared for what kind of data is going to be needed to provide the sufficient numbers of tests.

It is scary the number of tests that will need to be run for microarrays, according to our statisticians. Obviously industry should have their statisticians deciding what they think the right answer is so that we can have an appropriate discussion.

Tony spoke about the burden of validating reagents and bringing them to market. The burden of proof is on the manufacturer. On the other hand, we do have statisticians who say, I'm sorry, we don't believe your method was statistically sound or statistically robust enough. There will need to be interaction.

MS. PATTISON: Paula Pattison again. How about considering some of the data coming out of other markets, the European markets for example?

MS. KOCHMAN: FDA does accept foreign clinical or field trial data, providing we can have assurance that we can fully understand what was done, how it was done, that it was done in a way that is going to be the same as is going to be done here; if the populations are similar.

One of the problems that we will likely run into is that we do have a different population distribution here than some of the other markets have. So, while it is feasible to bring a product to market in the U.S. with only foreign data, it is not always possible to do that.

DR. WESTHOFF: Neil, would you care to share with us what your statisticians decided?

DR. AVENT: Well, we have 40 different tests for each SNP on the BloodChip array. At the moment we feel one test is adequate on the array.

From what Sheryl was talking about, I think that really applies to expression profiling on arrays where large numbers of tests will need to be done to access the expression differences between say one leukemia and another.

But from the BloodChip platform, we believe only one test is adequate.

DR. WESTHOFF: --- heterozygote?

DR. AVENT: Yeah. We can score a heterozygote.

MS. KOCHMAN: I'm sorry. I don't think I understand what you mean by one test.

DR. AVENT: For each donor. Just one test.

MS. KOCHMAN: Okay. But how many tests -- how many chips need to be tested against how many donors to prove that you have got confidence in the results?

DR. AVENT: Well, the C-marking requirements saw that we do 1,000 for KEL, 1,000 for Rhc and 3,000 for ABO and 3,000 for D.

MS. KOCHMAN: And do you know where they got those numbers?

DR. AVENT: No.

MS. KOCHMAN: I have a feeling I know and --

DR. RIOS: I have a question following up this. I look at your numbers, and I was wondering if it is 1,000 of each phenotype or if it is overall,

because my question -- of course, when you say these many D, you have so many D variants that you have to know how many from each allele or each variant you are doing for you to prove robustness and confidence.

So, did you break down in your variants, in your --

DR. AVENT: If you look on my slide, there is a certain percentage of weak D's that are required amongst that testing, and that is done for the provision of new serological reagents in Europe. So, we are allowed to use the same provision for a new genotyping reagent in Europe as well.

MS. RIOS: So you are not required to test them all in set numbers to make sure you get them all right or the percentage of time that you get accuracy in the data? How do you gather the information to prove that your chip is covering the whole array of gene expression variations instead of combinations?

DR. AVENT: It has to mark with -- you have to type the blood with C-marked serological reagents. So those serological reagents have gone through the same process that we are using for genotyping. So, for 1,000 samples that we have currently done, for example, for KEL, they have got to be typed with C-marked KEL typing reagents.

And any errors that we get, any discrepancies between serology and genotyping, we have to explain, whether or not it is just a standard clerical error or this is say a new KEL allele that no one has known about.

DR. RIOS: Now I am going to paraphrase Christine. With all due respect, I am talking about RHD and the ABO and I am leaving the other systems aside. It is a lot more complex, and I would like to come back to Marion mentioning that the ABO and D should be tested routinely by serology.

And I think this is a statement that is very profound, and I was wondering how that conflicts with what you are trying to do there. And how would one assess the best usage of moving to DNA typing in which circumstance? What is the effectiveness and reliability that we can draw from these data analyses?

DR. AVENT: I think that the number of RHD alleles -- I think we -- I say we scratched the surface. I'm not so sure about that. I think we possibly made a fair progress on the number of alleles. There is a finite situation that will be arrived at. Perhaps we will take 10 years to get that. Perhaps we will take 20.

But the genotyping platform will be able to predict very precisely what those alleles are once we know the molecular basis of them, and the testing platforms that are being developed have the capacity to type those new alleles, although it is not beyond the scope of the testing platform at the moment.

DR. REID: I think the technology is here yesterday. Actually, yesterday. Not today. For the minor blood group antigens. I think Rh is on the horizon, but it needs stuff that we have been talking about.

We need a lot more data first to be clever in the way we -- how many SNPs we put on the platform and how that is interpreted, but the knowledge is pretty much here. And in ABO, for the reasons I gave on the slide, for routine testing I think it is going to be a long day before I would want to receive a unit that was genotyped.

DR. FIGUEROA: But I don't think we are ever going to be able -- if I understood your lectures correctly, that we are going to ever be able to test for a clinical trial everything, because you have a mutation here, a mutation there that causes the expression of a partial D or whatever that is not always the same.

And if we enter ethnicity into that, then we are multiplying those factors. So, we need to be realistic on what we can expect from those reagents and what we can ask a manufacturer to test, because if we are going to ask to test for every single polymorphism or whatever, they will never be able to put anything out in the market because it is not possible. Or at least I think it is not.

MS. KOCHMAN: I think I mentioned that we recognize that we may not be able to wait for a perfect product to bring it to market, as long as we can convey what the limitations are and how to use it properly. So that is another area that I think will fall to everybody in educating the users about what the limitations are and when to know that something is different than it should be and needs further investigation by a reference laboratory.

I certainly don't envision that we will be waiting forever for the perfect system, but we need to understand what the imperfections are so we can convey them.

DR. JOANN MOULDS: I want to come back to the control issue because I think, Sheryl, you may have misunderstood. I think you are thinking about validation type controls.

I'm talking about the workshop recommendation that a water control, a negative control and a positive be run with each run. How do you do that on a multi bead microarray type thing? You cannot do that for 28 donor genes.

MS. KOCHMAN: I don't know the answer. I didn't answer because I don't know the answer.

DR. JOANN MOULDS: I want to ask the committee. Are they going to revise that recommendation or have thought about how this is going to be done?

DR. DENOMME: I can speak on a smaller scale. What we did was on each chip we ran a known control for the 12 SNPs that we were doing. Now, the complexity is a lot higher, but we have the capacity to take then 12 samples that were heterozygous and use up that portion of the chip for those controls, and the instrument itself needed some controls or another 12 there.

So the entire chip, one 50th of it, was taken up with controls. So these chips can get very large or the dots can be very small.

DR. AVENT: I think on our chip there are a lot of controls there as well, and the multiplex itself is controls there as well. And there is always a positive DNA control with the kit that you have to run with every sample to allow -- to check that the test reagents are adequately performing in the kit.

So, that is a control that needs to work to be able to score the results, and some of the failures that I showed you in ours was because of the failure of the internal control. There was an issue with the internal control.

So, the QC is actually quite careful and will not allow you to score a correct result, even though you may feel the genotyping of your test sample is perfectly adequate.

DR. DENOMME: To give you an idea, each well of a 12 SNP microarray had four controls that were an instrument control alone. So, there are actually 16 dots, but four of them cost you to get an accurate result out of that well alone.

Then the chip itself had -- from the start of PCR to the end had 12 controls and then there were the 12 SNPs themselves and we had heterozygous samples. So we lost another 12 wells out of 384 to that process as well.

So, it is a little bit complex, but you can handle it both within a well and within a chip. And then, of course, the machine does things like at the outside of the chip for higher frequency errors, suggesting that there is something wrong with that chip during analysis, and it can even be rejected on that basis. So, it is quite complex.

MS. LOMAS-FRANCIS: Just to come back to your question though, Joann, with regard to the recommendations that came out of the ISBT workshops, those original recommendations in 2004, or suggestions, were initiated based on manual testing. I think even in 2004 nobody had an idea that we would be sitting where we are currently.

And in the 2006 workshop there was some testing submitted done on microarray systems, but that really didn't figure in those results extensively and the majority of people are still going to be using manual testing. So, there was a little bit of discussion as to whether or not people would be implementing microarray technology before the next workshop.

And, you know, the answer was that most people who were at the Cape Town meeting were not going to be in a position to do something like that. So, those recommendations are still very valid for testing.

But I think your question is very interesting, and it is a question that will have to be answered by the manufacturers of the defendant array systems. They will have to, in the first instance, convince us that those controls are there and that they are adequate.

DR. REID: I was going to say, Ghazala, why don't you tell us about the controls on your systems.

DR. HASHMI: Ghazala Hashmi, from BioArray Solutions. I think what Joann -- if I understand your question correctly -- is trying to ask is that if we are running a test on microarray, such as a BioArray chip, and you are analyzing for 28 antigens, do you need a positive control for each antigen?

What we do is we run several positive controls and what they do those will be say positive for Fya and Jkb and KEL and, you know, duffy and something like that. But do you need a positive control for each and every antigen? That is the basic question.

You can have positive controls. We have certain cell lines that we run right now, five or six cell line samples, and you will have positive controls for it. But do you need to have homozygous A allele, homozygous B allele and heterozygous alleles for each of the antigens that you are screening for?

That is the question for FDA to answer basically because there are -- cell lines are not available right now or the positive controls that you could use for these analyses.

DR. BELLISSIMO: Dan Bellissimo. I just wanted to point out there is some precedent for some synthetic controls that are being developed for cystic fibrosis, which there are panels being run. Anywhere from 23 mutations up to 70. And I will discuss this a little bit tomorrow in my talk, but there are synthetic controls being developed that contain all of the mutations and everything that can be used as a positive control to fully check a chip or a bead array all at once.

DR. AVENT: Those synthetic were used in the development of BloodChip. That is what Progenka did.

MS. LOMAS-FRANCIS: Actually, I was just going to come back. I

get the sense that probably one of your main concerns is coming back to the water control and to make sure that the system is not contaminated by random DNA.

DR. JOANN MOULDS: Right. We do that all of the time. But if FDA is going to require that we do a positive control for each system, we will be running 28 chips to do an eight chip run. That seems ridiculous and that costs would be phenomenal, and you certainly won't be using it.

For those who aren't aware of the B chip, you do them as allelic pairs. So you are doing K and k. You are doing Fya and Fyb; Coa and Cob. So aren't you, in essence, kind of controlling? If you get a Coa negative, it better be Coa positive? And if it is negative for both, then I certainly would investigate that for a null status or something like that.

MS. LOMAS-FRANCIS: Yes. I think you have just answered your question as far as that is concerned. And I suspect in the beginning, if you are talking about something as rare as a Coa negative, we are going to be checking that serologically. But we are only going to be testing that one sample. We are not going to be testing the 100 or the 1,000 donors with anti-Coa. We then will have the Coa to check.

MS. KOCHMAN: I think you all need to remember that you also need to worry not just about what FDA is going to require, but what CLIA. We try to make sure that we know what is one CLIA's mind. We like to say the same thing since we both work for the government.

I would just say that it is not something that I am prepared to just spout off and answer.

MS. : What does CLIA require for HLA?

MS. : I'm not an HLA person, but I think it is actually regulated.

(Pause.)

MS. KOCHMAN: Well, it is 5:10. I was told they would shoo us out of here if we hung around much past 5:00. I thank you all for the lively question and answer. It was the kind of thing I was hoping for. Again, more questions than answers.

Okay. We will see everybody back here again tomorrow.

(Whereupon, at 5:12 p.m., the meeting was recessed, to reconvene September 26, 2006 at 8:30 a.m.)

Cover Page

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I N D E X

Workshop on Molecular Methods in Immunohematology
September 26, 2006

Facilitated by Sheryl A. Kochman

I N D E X (Cont'd.)

KEYNOTE: “---” denotes inaudible in the transcript.
“*” denotes word was phonetically spelled.

P R O C E E D I N G S

(8:40 a.m.)

Opening Remarks

by Sheryl A. Kochman, Facilitator

MS. KOCHMAN: Okay. So we are ready to resume. Yesterday we kept on time really, really well, and I want to thank everybody for that. I do have a couple of announcements to make this morning. If you need a shuttle to either of the airports please see Wanda Dawson by about lunchtime so that she came make those arrangements for you, and also there is an evaluation form. It should be in the lefthand pocket of your handout. We would appreciate it if you could fill that out and give us your feedback on the meeting.

We are going to start today off with a presentation from Marion Reid talking about the Consortium for Blood Genes and how it came into being and what it is all about.

Consortium for Blood Group Genes - CBBG

by Marion E. Reid, PhD, FIBMS

DR. REID: Good morning. So the Consortium for Blood Group Genes or, as I will call it, the CBBG. I want to thank Sheryl for asking me to give this talk because I

was getting a little despondent about the whole thing, but I then I realized just how much we have achieved in two years. So it changed my attitude, and everything is about attitude, so I thank her for that.

(Slide.)

So I thought I would tell you how it got started and a little bit about the original name, what the purpose is, what the accomplishments have been, and what the mission and goals are -- not necessarily in that order.

(Slide.)

Actually Greg Denomme reminded me that it came on a wave of a couple of successes. One was the book called Molecular Protocols in Transfusion Medicine, which was coauthored by Greg, Maria Rios* and myself; and it had sold 500-plus copies by 2003, which we thought was a pretty good number considering it was such a niche market -- back then anyway.

We had developed, or I had developed, a proficiency exchange program for DNA samples because I was concerned that we were doing this testing and had no way of doing a proficiency or quality assurance. So it was set up that on the change of the clock once a year I would send DNA samples to all the participants, and then like in the spring

and then in the autumn all the participants would send a sample to me so that we would control each other twice a year.

There were and are eight participating labs. Well, to start with actually it was Dan Bellissimo and myself, and then as people became aware of it it expanded. So it now includes Connie Gayle, Jeff in England, Greg in Canada, Jill in Sweden, and Lillian in Brazil, so it is international, and we just send some very simple stuff. It is designed to pass, not fail. So we sent DNA from one or two samples to say which SNPs should be tested. So we would say for FY or for JK or something like that. Then the results are sent back to the lab who sent the DNA, and then they confirm the serology, the typing from serological results so that we have both got documentation. So we are controlling. The testers control the lab that sent and the lab that sent controls the testers. A little strange, but it works and it is very little effort to achieve.

(Slide.)

So how did the CBBG get started? With that as a background, and then in 2004 I was fortunate enough to be speaking at a symposium in Brazil that was about molecule aspects in blood transfusion; and I came up with the idea of

having a support group, that was developing and that we could help each other.

(Slide.)

There is me talking and there is Marisa talking. We are very serious and we got into some interesting discussions.

(Slide.)

Then I went on a vacation, relaxed, enjoyed myself, and did usual things in an unusual way. There is me snorkeling, but I am towed behind a boat so I do have to do any of this leg flipping or hard work.

(Slide.)

Then I went back to Campenas and was hosted by Lilian, and for breakfast we had stuff that was just very related to immunohematology. We had --- and --- and ---.

(Laughter.)

So when I mentioned this idea to Lilian she was supportive and encouraged me, so with my usual enthusiasm I continued.

(Slide.)

At that time we thought that America's Association of Blood Group Genes would be a good name because we knew that the Europeans were already gathering themselves and

didn't need our help. So we called it the AABGG, and then when I returned to the US I contacted Connie and Greg because I knew that they were doing molecular analysis of blood groups for clinical purposes, and they also thought it was a good idea and came onboard.

(Slide.)

So I have put structure, and that is a little constructured. Put me as a coordinator, Lilian as the liaison for Latin America, Greg the liaison for Canada, and Connie the liaison for the USA, and obviously we are volunteers and everybody else who is in the group. We are there to help each other, and it is everybody in it together. It is just somebody has to lead the group. So everything I am going to say from now on is the group that has achieved it. It is not me. I am just the spokesman for CBBG.

(Slide.)

So in the fall of 2004 we contacted people that we could think of who might be interested in molecular analysis for either blood groups or platelets and we asked if they would be interested in joining a consortium and asked them if they knew of anybody else. So we sort of put the network out there trying to get the names of everybody that might be

interested and we created an email address of 21 people. I sent out an email outlining the purpose of the group and received a positive response from everybody. It was 100 percent response, which is pretty impressive. We arranged a meeting to be held in Baltimore in October, ad that meeting was attended by 24 people.

(Slide.)

We made some decisions at that meeting. The language would be English, which is fortunate because I don't speak anything else.

(Laughter.)

The membership is open to -- if membership is the right word, is open to anyone and everyone who is interested in DNA analysis in blood transfusion. Obviously the focus is on red cells, but we are also open to platelets and neutrophils, the AABGG name was changed to Consortium for Blood Group Genes so that there was no implied geographic restrictions to membership. We didn't want it to be only America's, but that is our focus. But it doesn't mean to say that anyone else in the world can't come and help us and we help them.

So we decided that we should meet where members might be attending meetings anyway so their hotel and travel

was already paid for, such as before or during or after the AABB, ASH, or ISBT meetings because we have absolutely no treasury. There is no money. This is all done by individual people paying whatever it is that they need to pay.

The idea was to interact, share knowledge, ideas and concerns, and to identify formats for interacting. There were the obvious ones. We thought of email, newsletters, websites. We did achieve a name. Sergio Taloni paid for and applied the name cbgg.net, and we also thought of the AABB special interest group, or the SIG. The disadvantage of that being that you have to be a member of the AABB, so it could be a little restrictive, but those were some of the thoughts we came up with.

(Slide.)

So at the first meeting we thought it might be useful to write SOPs, standards, and prepare templates of request forms, worksheets, reports, and disclaimers so that were all sort of in the same ballpark and doing something that made sense and weren't overlooking something obvious. We were to establish and operate a proficiency program, to establish a repository of well-characterized DNA for assays, validation, and for controls, to identify sources of

funding, to identify centers of excellence for referring unusual samples for detailed analysis of genes. So if you find something unusual, which lab is good at looking at that particular variant. Like in the good old days if we had something rare we would have sent it to --- Sanger. So to identify those labs that are willing to do that extra work, and we thought we would write a letter to the editors to advertise that we are there and again put out there so that if anybody is interested they can join us. It is not just an elite club.

(Slide.)

The areas of focus are actually pretty much what I just talked about, but there were working parties established of -- you know, this is a small number of people, so you have one or two people in each working party, and there was some overlap. They were working on the disclaimers, the DNA repository, funding, proficiency programs, the forms, the SOPs, standards of practice, structure and bylaws, terminology, and website.

(Slide.)

So then I got brave and I thought, well, you know, if we are going to start this technology we cannot go anywhere with the FDA. They are going to either have to

approve it or indicate that it is appropriate. So I spoke to Sheryl and asked whether the FDA would be open to considering or allowing or embracing the molecular methods for testing for blood groups; and she was extremely receptive and encouraging and suggested that there should be a meeting of interested people to discuss the needs, the value and the issues, and here we are. So, thank you, Sheryl.

MS. KOCHMAN: Thank you.

DR. REID: Greg wrote a letter to the editor. It was published in Immunohematology in 2005. The content was describing what happened at first meeting, a little bit of background of why we thought it was needed, an overview of topics that were discussed -- you heard that list, and asked for anybody that was interested to get involved. We heard from nobody, which I was initially very disappointed in, but then I got quite excited because I figured that we had really reached out in that original casting our net and we had captured everybody that really wanted to be involved -- or nobody reads Immunohematology.

(Slide.)

So the second meeting was held in Seattle. There were 19 attendees, and the discussion points included

misconceptions about DNA testing, genotyping for blood group genes not being a disease, storage and unlinking DNA samples, the terminology of nucleic acid testing versus DNA testing. A mission statement was prepared. A logo was designed prior to the meeting and voted upon, and working parties broke into small groups to work.

(Slide.)

The mission statement is to establish standards, operate a proficiency program, and provide education for laboratories involved in nucleic acid testing for the determination of blood group and platelet antigens. There is no indication of superiority or inferiority. It is just that is what they were doing.

(Slide.)

Here is the logo, and I thank Lilian and her colleagues in Brazil for playing and tweaking and doing this. So we have indicated the three countries that were initially or are the liaisons, but the DNA is sort of connecting us, but going on around the world indicating that anybody who would like to join is welcome.

(Slide.)

The third meeting will held in Miami Beach on October the 19th, and we have decided that instead of

focusing on too many things, which is overwhelming, that we would focus on a few selected items. So to help us identify which were the most important, we emailed members for their priorities. We listed the working list that you saw earlier, and the majority responded with their preferences, so the winners will be discussed. I haven't yet analyzed those responses, so I can't tell you what they are. The agenda will be decided not because of this meeting, but, you know, there is only so much you can do in a day. To date we have 18 -- or it is 19 now. I just heard of one more yesterday. So 19 members plan to attend, and we have had eight regrets.

(Slide.)

So the goals, the primary goals of the CBGG: to establish a DNA bank; to establish and operate and proficiency program; to develop template request forms, worksheets and reports; to develop disclaimers; and to develop standards of practice that we can follow.

(Slide.)

So the CBGG is all about helping each other. It is going between everybody, just an interactive communication. So you might think there is a core expertise in the DNA repository, discovery and research can be one

little area. An advisory panel or board, standards or practice, proficiency programs, and participating labs with clinical results and proficiency participation.

I think that is it. That is my usual, you know. DNA is not going to replace hemagglutination soon, but I think the pair of them together are very powerful. Thank you.

(Applause.)

MS. KOCHMAN: I forgot one of my other announcements. A number of the presentations for today are not in your packet, but they are available out at the registration desk. So you can pick those up during the break on your way, however you want to do that, but they are out there.

Our next speaker is Dr. Ghazzala Hashmi from BioArray Solutions, and she is going to talk us about blood cell antigen determination by DNA analysis.

HUMAN ERYTHROCYTE ANTIGEN (HEA) DETERMINATIONS BY DNA ANALYSIS

Ghazzala Hashmi, PhD

DR. HASHMI: Good morning. I am happy to be here this morning. Thank you very much, Sheryl, and FDA for organizing this meeting. So what I am going to do this

morning is to start with a brief history of BioArray Solutions. BioArray Solutions came into existence in 1996. For a while it existed as an R&D organization. Platforms were developed and an integrated system was established. In 2001 BioArray Solutions was incorporated, and then we developed several applications on --- platform. Some of our initial applications included the genotyping of inherited disorders such as --- Jewish diseases, cystic fibrosis and others, and also actually typing, actually DNA typing.

We were introduced to blood group DNA analysis by way of scientific collaboration with Dr. Marion Reid. In around 2003 that evolved into the development of our current HEA --- format. Since then we have worked with several leaders in blood centers in immunohematology labs, some of them are present here in the audience, and also in hospitals. So today I will highlight two of those collaborations. One with --- blood center with Dr. Marion Reid on donor site and another one with Montana School of Medicine with Dr. Caroline --- where we looked at the patient population and how the DNA analysis can be used to predict antigen typing.

(Slide.)

After yesterday’s talk I don’t have to tell you

how DNA analysis can be used for antigen typing. We had very good discussions about different techniques that have been used, SSPs, PCR, RFLPs, and also microarray analysis. The application of DNA analysis is not new in immunohematology and has been used in various labs for confirmation or for antigen typings and also for difficult cases. But most of these techniques, as we also heard, are manual like manual PCR followed by RFLPs or sequence-specific primer PCR. So now the current understanding is the DNA technology is a useful tool to be used in immunohematology and can be used for used antigen prediction.

So what is the next step what to do? A current practical limitation would be the manual nature of that and if you doing it in donor centers it is hard to do high throughput analysis or larger scale screening of the donors, and also with the patient population it has to be quick assays, quick as a platform where several antigens can be analyzed simultaneously and is in a short period of time.

So that is where we come in, so what we actually -- we are not really making anything new or developing anything new. What we did is to take that, all that information, basically SSPs, and what we are doing is we are

putting it on an array format. The other format that we use is bead-based assays, so these are also standard. Bead formats are the standard format used in molecular diagnostics, and what we are doing, we are putting those beads and assembling it on the microchips that are silicon wafers and doing the multiplex analysis on that.

So --- beadchips are different than the current understanding of microarray. When you think about microarray it is the glass slide where you have several probes that are linked, grown on the surface of the glass slide, and usually the understanding is when you think about microarrays is the expression analysis. We have Affymetrix chips, we have thousands of genes are screened simultaneously, and of course at the end the understanding or the general concept is that at the end there is so much data that analysis is very different. So these microarrays are completely different from that, especially other microarrays or bead arrays. We have our --- on the surface of a three-dimensional structure that is bead, and then beads are going on the surface of the chip, and of course I will describe it in detail.

(Slide.)

So what I intend to do today is to give you an

introduction about our technology. So what are the beadchips, method of analysis, and then HEA analysis and how it is done for patient and donor population, and then summarize the results.

(Slide.)

So how do we make the beadchip? So as you can see, we start with a bead that is color-encoded, and then the beads are -- so there are several different colors. We have more than 100 colors of beads now. We synthesize our own beads at BioArray Solutions. We have manufacturing facilities and we also color them in house, so we control all the processes in house. Those beads are then functionalized.

What functionalized means is that when you select an application, say if I want to a cystic fibrosis panel, I have 25 mutations, I will take a number of beads that is enough to do 25 mutations and then for each mutation there will be a different color of bead. So it means that for each probe or each --- there is a specific color, and that color is the address of that probe for downstream analysis. Then these beads are coupled. After coupling these beads are pulled together in another chamber and then assembled on the surface of a silicon wafer. This is a six-inch silicon

wafer, and it is microfabricated just by standard methods. That is in the industry using industry right now.

What we do, we microfabricate them in 1.725 millimeter diameter for a chip, but only a small area in the center of this chip is our bead chip or the array, and this area is only 300 microns. So we can make 5,000 chips like that, and each chip, that is 300 microns in diameters, has capacity to hold 4,000 beads in there. So for each application there will be a separate, a different -- so at this point everything is the same. It differs at this, so you will have pools of different applications -- cystic fibrosis, HEA, HLA -- and then the manufacturing is of course a separate process.

(Slide.)

So how these beadchips are used. For using the beadchip I highlight an example of antibody profiling. In this case what we do is that antigen is coupled on the surface of a specific color of the bead. Then these beads are assembled the way I just described. An image is taken of that array. This image is called a decoding image. This image is stored during the manufacturing process and it is unique for each application, so therefore the position of each bead, you know, the color of each bead as it is linked

to its --- is already in the database.

What is done after that, the plasma sample from patient or the donor is applied on the surface of the beadchip that will binding of the antibody on the surface of the bead, and then this antibody is detected by a secondary antibody by amplification. Then you will take that.

So that this part is done is done in house. This is the manufacturing process. This is the user part. So what a user is doing, when they are getting these beadchips they are performing the assay and taking just a single image, and this information is already stored in the database that is provided with each beadchip format.

So what will happen in this case is whenever there is a positive signal you will have a fluorescent bead there. If there is a negative signal there is no fluorescent bead. What the software does, that method of analysis is it will take this image off the array assay made and superimpose on the surface of the beadchip. That information is already there. It will find the position of each bead and calculate the signal on each bead type and then give you the result.

This was the formula that was used in one of applications of auto-antibody profiling, and for that we received FDA CDRH clearance in 2001. During that time since

our --- system is the same. So the platform that is used for protein typing or for the DNA typing is the same, so we do have a clearance on --- instrument array imaging system.

(Slide.)

So how do we use it for DNA analysis now? For DNA analysis, for example, we are doing antigen analysis. So you will take the donor or patient sample, DNA is extracted from that. We use current protocol, use only 200 microliter of DNA, and we eluded around 50 microliter of volume. Do a multiplex PCR. For HEA I will discuss it later in detail. There are 18 plex PCR, and then there is post-PCR processing. What post-PCR processing is doing is actually making a single-stranded target from the double-stranded one, and then you apply on our beadchip format that has all the probes already assembled on that. So --- if you are doing a 96-sample result for HEA --- 28 antigen result is available.

(Slide.)

The overall protocol is shown here. So this is the multiplex PCR, post-PCR where you produce single-stranded DNA molecules. DNA goes on the surface of the chip, elongate, and you get an assay made. The basic principle is exactly the same. There is a decoding image

already present in the assay in the database and the assay --- that will overlap and give you a signal.

The overall process takes about less than five hours, and in house we did a time motion study with single --- and single instrument, and we found that 200 samples can be done if you start 8:00 in the morning. 5:00 in the afternoon 200 samples can be done completely. So it means at the end of the day you can you have 200 samples with each sample with 28 antigen information. This throughput can be of course increased if there are more instruments or more manpower is available.

(Slide.)

Okay. Now how is it the DNA is actually analyzed? So one of the things that I didn't mention in the previous slide, how the discrimination, how the allele discrimination is achieved in these cases. In our analysis it is not based on hybridization. That is mostly done in microarray format.

What we do, we basically as I said before in my introduction slide, we are taking the SSP primers, if you will, and putting them on the surface on the bead and actually we are doing a mini PCR on the surface of the chip. So the discrimination is very, very high, so as shown here that is the bead that is linked to each probe is linked

here, and the --- assigned target would bind here and elongate if there is a match. The process of deduction, we call it EMAP, or elongation --- analysis of polymorphism, and that means that you are -- the discrimination is very high and it is not based only on the --- of two DNA molecules coming together, but on the specificity of the DNA polymerase. That is very, very specific for any mismatches at the three --- end.

So just in a little bit more detail for each, --- for example in each HEA typing is I am going Fy^{ab} antigen. So there will be one color for Fy^{a} and another color for Fy^{b}. Te probes are exactly the same in sequence except at the three --- end, and that is decided on the basis of the SNP that is linked to that antigen expression on the red cell. So when you create your single-stranded target and add it on the surface of the beadchip, a single-stranded target will bind it, an enzyme will bind, and also when the other EMAP makes an elongation it is just like in PCR, includes the DNA polymerase plus DNTPs and --- chloride. You know, the usual PCR mix. Whenever it sees a mismatch there is no elongation. If there is a match, perfect match, it will elongate. And since there is fluorescence present there in those DNTPs, it will fluorescently label that bead

and that could be obvious when you take the ---, and when the data is calculated.

Another point I want to make is that redundancy of the probe molecules. So there is another difference between the other microarrays, that in other microarrays it depends how many probes you actually put that are coupled on the glass surface. What we are doing, we using the number of beads. The number of beads is from 50 to 100 beads of each color are present there, and then for each bead type, for each bead, the surface area as compared to the DNA molecule is huge. So each bead can have up to 1,000,000 probes attached to it.

So if you put all that together, there is very high redundancy present there, and that creates very high discrimination for allele discrimination for data analysis. Statistical reliability is already built in the system.

Another thing I didn't mention is that, you know, with the specific group there will negative, seven negative, and positive controls are also added. That is a different color bead that is added on the pallet. So what happens here is that when you see a signal intensity, this a mean signal intensity on that say 80 beads and each bead of Fy^a and 80 beads of Fy^b, and each bead has 1,000,000 in that.

So you have a mean intensity of allele A and allele B, and then we do the data analysis by calculating a discrimination ratio.

(Slide.)

So this is how the image is recorded or data is recorded. So what it is doing, we have our array imaging system that is basically a fluorescent molecule that -- excuse me. Fluorescent microscope that has the filters are -- it is modified with filters and it has software of course. So what it is doing is that when you load your chip this is a --- format for a 96 --- plate, and what you have is that each -- this chip carrier has a barcode attached to it.

So the user will after you perform assay you will put this chip carrier on the surface of -- just like on --- focus the first chip. Once the first chip is focused then the whole operation is automatic, and then it goes from chip number one to chip number 96, and each step taking a single snapshot. Since we have --- the size of the array is very small, only 20 microns, you don't have to obtain the signal intensity separately. So it is just a simple operation where you take the image, a single snapshot of the whole array and the image is analyzed, and then it will produce

just like I said before, an assay image; and then for the analysis part it will bring -- the user will get this information for that bead chip carrier which they are receiving, and the analysis will perform by overlapping the signal on these beads on the assay image by overlapping it with a decoding image.

(Slide.)

For data analysis as I said before, that this is a mean intensity on each probe, and you see the two are elongated. This is just the raw data. Two are elongating. It means that it is heterozygous. When only single is elongating it is the homozygous. So for what we do, we calculate the discrimination ratio of allele A and allele B and then a value discrimination ratio or a delta value is created of course by after the background normalization. Then when these --- are generated for each --- or for each SNP, and when they are plotted on a graph it will create tree specific clusters, so this cluster, AA allele A homozygous, allele B homozygous, or AB heterozygous.

There are grey zones, are also present around these clusters. So if any value is something like this fall into these grey zones it will be flagged as an indeterminate call and the user will have to go back and repeat that.

(Slide.)

There is no manual transcription needed here, so once you load your sample and you scan the beadchip barcode, so all the information is in the database. So this is an integrated system as I said. We have several different applications that use the same integrated platform. So it starts with the image's acquisition with --- imaging system that will do the -- then it goes into the database where you will have the decoding images and image analysis is done, and then genotype or alleles are described on --- in our application server. That is all done in the same application format. Then for HEA typing for mutation analysis a mutation report is generated. For HEA what we do, we convert that genotype or the DNA analysis on each SNP typing into the allele assignment and provide a phenotype report for the user.

(Slide.)

For HEA panel for blood group genotyping this is our panel. This is our current panel that analyze 11 blood group system and 28 antigen in single chip format. We also included one polymorphism for hemoglobin S. So when a sample is analyzed in blood banks or in donor centers or in hospitals that the analysis of whether that donor has

hemoglobin S positive or negative is also determined.

(Slide.)

So how the genotype to phenotype or the DNA analysis with the data you are collecting on the basis of SNP is used to determine the phenotype or antigen prediction is just by simple look-up tables. So these look-up tables are part of our software, so I am just describing that to make it, you know, obvious that as it was said yesterday also that most of these antigens are one-to-one. So there is a single SNP as our current knowledge is today, that a single SNP is responsible for single antigen as shown here. For example, for KEL there is a single --- AA. You will get a SNP typing, AA, AB, and BB. So AA means that big K is positive, little k is negative; AB, big K, little k both positive; BB big K negative, little k positive. And it is true for KEL, Kidd, Diego, Colton, Landsiener, Lutheran, and Scianna and also for our hemoglobin as mutation. For hemoglobin S we can say that either sickle trait negative, sickle trait positive, or it is HPS homozygous. So this is a condition when there is only single SNP is responsible for antithetical antigen, but there are certain cases where more than one SNP is needed.

(Slide.)

For example, in duffy --- case. So in that case the --- present there. When there is a situation the software will go and find any -- you know, on the basis of the DNA typing find that combination and then give a phenotype report. That is also true MNS here. It is actually showing only MNS, but there are silencing mutations that could affect the expression of big S --- antigen, and those polymorphisms are also included.

(Slide.)

The phenotype report that is generated, it looks like this. Just like the phenotype report you are used to seeing by red cell typings with all the antigens are listed on the top. This is our chip ID, meaning that this HEA are a specific, unique number. For a 96 --- plate it will be the same number for all 96, only the position of the well is different, and then there is a field where the user, if like to, add their specific sample number. If you have --- format that could be --- and it will come into this phenotype report; and if there is anything wrong during that assay, say high CV, high background, there is no signal, --- dropout, that will be shown here. As you can see here actually I listed their silence and whether silence is present or not. Now in our current format this field is

already integrated in here and that, you know, the software will take care of that and just give a typing on the basis of if the silencing is present or not.

(Slide.)

Okay. So for HEA phenotype by DNA analysis of a current --- we did a large-scale study with Dr. Marion Reid's group at NYBC and we submitted a manuscript in Transfusion. I will just discuss some of the results. During this study we have New York City donors. There were 2,355 donors all for self-identified ethnicities and they were screened on a beadchip panel. At that panel we did not have silencing mutation and also -- C and E present on that.

In our analysis we had in New York Blood Center they had the phenotype information. These were partially phenotyped, meaning some --- have phenotype available for Duffy, some have available for Kel, Kidd, Dombrock and MNS, and there were 4,534 phenotype information available. We found 4,510 of them are concordant and they are listed here. There were 24 phenotypes that were not concordant. So resolve that we did the sequencing of each amplicon and Megan's group did the RFLP on those samples, and we found that eight samples out of 15 for big S little s, eight

samples had silencing mutations. So that is why there was a discordant there, and since then we added those two mutations on our panel. There were the other ---. They were all resolved in favor or --- and we found that most of them were clerical mistakes or first-time donors that were not really conformed by CEDR* tying at that time.

(Slide.)

S0 in this instance we can that phenotype, that the DNA analysis can predict the phenotype for this set of antigen.

(Slide.)

During this study we also determined the antigen frequencies within these different ethnic groups, and as expected they were different between African-American, Asian, Caucasian and Hispanic populations. The biggest difference was noted in a Duffy and MNS group system.

(Slide.)

So during this study we also as I said there were 4,000-some phenotype information that was available, but by -- there were 19,000, more than 19,000 new phenotypes were also, antigen-negative phenotypes were also identified during that time, and they could be used for further -- you know, by confirmation by serology and could be useful donors

to support transfusion of matched units. Numerous examples of a rare configuration but also identified such as S, big S, little s negative, U negative. There were some U -- and U negatives, Lutheran A positive, big K positive, two samples like that, Joseph A negative six, and Colton B positive.

During that time we also did the time motion study as I told you. So now we can say that current protocol in practice, it enhances productivity in a person. A technical person can do more than 200 samples a day, and also this is an assay that is simple to perform that people with not too much molecular background can be trained very easily. We have seen several groups have come to our labs recently for training, and with a week of training in house at BioArray, and then they go and with some practice, a two-week practice, they are proficient to use the system in their regular DNA tying.

(Slide.)

Okay. Now just to see what is the clinical utility of the HEA typing for patients. As I said before, we had a collaborative product project with the Mount Sinai School of Medicine with Dr. Caroline Vincent* and initially what we did was selected a number of cases where we --- the

patients with the disease where the disease condition itself or the treatment of the disease might interfere with DNA analysis; and those conditions are listed here, such as cytotoxic drugs that can reduce the number of white blood cell count and also hemoglobin --- several transfusions. So we selected several different conditions, and some of these samples our DNA yield was very, very low, but we were able to amplify the full panel and analyze these samples correctly for as low as around three nanograms of DNA. So a total of around 20, 25 nanograms of DNA that was used in the assay so that our initial collaboration and initial study was very successful.

We feel confident that in the patient in the hospital in the patients that are going through different kinds of therapies it could be used efficiently for this kind of DNA typing. One of the important cases which has been discussed yesterday also in great detail is the multiple transfusion, a patient with multiple transfusions where serotyping is not that -- cannot be done or is not reliable, and also people with autoimmune hemolytic anemia.

(Slide.)

So I am highlighting some of those cases here. There were seven cases where we took the samples pre- and

post-transfusion and did the DNA analysis, HEA beadchip analysis on that. Then some of them were very massive transfusions. As you can see here, the liver transplantation has 74 transfusions, and these transfusions are turned very fast and these are whole blood transfusions; and then there were cases where sickle cell crisis, sickle cell anemia, and heart transplant patients that we did the serotyping and genotyping before and after a massive transfusion, and our HEA beadchip typing was able to identify the typing of the patient correctly in all of these cases. Then there are four cases I have highlighted here. Those have serial autoimmune hemolytic anemia that the serotype cannot be determined by phenotype method, and we were able to do that with these patients, too.

(Slide.)

All right. So in summary we can say that critical clinical conditions are so far with critical clinical conditions we have seen the DNA analysis or HEA beadchip analysis was not affected by that, and it was a useful tool to determine the phenotype or predict the phenotype on the basis of DNA analysis and also it permits the analysis on very small aliquots of samples. That goes for both small quantities and quality of the DNA and also a small volume of

blood, that as you all know in cases of newborn the blood is a very small volume of blood is available. Even after massive transfusion the valid phenotype could be determined on the basis of this genotype analysis.

(Slide.)

So in summary we can say that our beadchip HEA analysis permits the reliable determinations for extended phenotypes as shown by a large-scale screening for patients and for donors with diverse ethnic backgrounds; and the current protocol in practice, as I said before, enables technical personnel to perform complex tests by minimum training, and enhances productivity because several sample could be analyzed simultaneously in one day.

(Slide.)

So what are our next steps? As I am sure you noticed, yesterday we had a lot of talk about Rh. In our panel this is a minor blood group panel, and we have only two markers. One for CEC, big C, little c, and big E, little e; and as you know there are issues with big C typing, sometime the variant typing, and so we are in the process of developing an Rh variant beadchip that will analyze Ce variant and also D variant. That is most clinically significant. HLA as I mentioned is already being

used as already being used a DNA analysis, and we have very well established allele assignment programs that can be used for HLA class one and class two types; and also HPA is our most recent reject that we are working on, and it is in development.

(Slide.)

All right. So if you need more information or a copy of my presentation please write to me at that address, and if you have any questions I would be happy to answer to. I think we will do it at the end. Thank you very much for your attention.

(Applause.)

MS. KOCHMAN: Our next presentation may actually not come off. I am not sure. We were going to try to have Jill Storry give her presentation by phone. I am afraid she may have not been able to stay on the line, but I am going to try to get her right now.

(Attempting phone hookup.)

So we hope this is going to work. The one problem I think is that Jill may not be able to hear. We are going to have to take our questions after her presentation, and she may not be able to hear them, so if there are any I will repeat them. So your first slide is up, Jill, if you can.

Application of Genotype Analysis to the Quality Assurance of Reagent RBCs

by Jill R. Storry, PhD, FIBMS

DR. STORRY: Okay. Great. Well, I would really like to be there, but at least you --- my voice. Thanks to Mark Davis who is --- to phone up, so -- anyway, I am sorry I couldn't be there, but I thought what I would do really more to get discussion going because it is something that many of us have discussed before is to just review our little study on the application of genotype analysis to the quality assurance of red cells, reagent red cells. This is something I know that many labs have done, but I have just put a few slides together based on our recent paper.

(Slide.)

So the next slide it really describes the aim of our study, and it was to use molecular genotyping methods to examine our in-house test results, and here in Lund we have assorted in-house ---. We have a three-cell antibody screen. We have a four-cell extended antibody screen. The rules definitely seem to be a bit different, and we also have a couple of in-house panels drawn from our local donors.

So we asked the following questions: Are our test

red cels what they say they are? Can we miss potentially clinically important antibodies because we are using red cells we think carry double doses of a given antigen? Really I think you will see at the end of the presentation we haven't --- that. It is very hard to measure. And thirdly, do we need to change any of our test red cells based on the outcome of this study?

(Slide.)

On the next slide I have just shown the test red cell requirements according to the Swedish handbook for transfusion medicine, and as you can see they are very similar to many of the standards described by other European agencies; the UK agency, the German agency, and of course very similar to those described in the US. We are mandated to have as best we know double dose of the commonly encounter Rh antigens, D, C, little c, big E, and little e, Fy^a, Jk^a, and big S; and then on our screening cells we are expected to represent CW, big K and little k, Kp^a, Fy^b, Jk^b, and little s, at least in single dose, and then of course MP1 and Le^a must be represented, but that is the only requirement we have for those.

(Slide.)

On the next slide it is really just information

which probably this audience needs no clarification, but I will go through it anyway. Of all the antigen that we encounter in the immunohematology lab, the actions that cannot be categorically determined as carry double dose or determining the dosage at all are A and B actions, D, Fy^a and Fy^b, and of course that is because, as shown in the box beneath, a person of group A can be genetically homozygous for the A gene or can be heterozygous for A and assigned O allele. Similarly with D-positive what could be homozygous for a normal RHD allele or one of many other combinations of RHD with a partial D or more commonly RHD ---.

When it comes Duffy A and Duffy B then there are two major silencing or muting alleles in that system. A person that had double doses for Fy^a can be homozygous for the Fy^a allele but may also be heterozygous for Fy^a with a silent allele or Fy^b with an Fy^x or even Fy^b allele, and that is a lot more common certainly in our area of the world. Conversely, you might expect for a person appearing to be double dosed of Duffy B can be homozygous for an Fy^b allele or heterozygous for Fy or Fy^x and this is very population-dependent as you all know, so in the European population we are concentrating or expecting perhaps wrongly to see a higher occurrence of Fy^x. But sort of the migration of the

world populations I don’t think we should be too rigid about what we expect to see and not to see.

(Slide.)

On the next slide it just shows can we improve -- titled "Can we improve the profile of our test red cells?" I think the introduction of molecular techniques in the last year or two is we certainly can improve or increase the information by DNA analysis on those antigens for which there are no or few antisera available, and very obvious examples have been described by us and many other people. The antigens of the Dombrock system initiated by the New York Blood Center, and also antigens that are a practical clinical relevance in some countries other than others. For instance, Di^a and Di^b, and these are antigens where the sera is not readily available commercially and it is really -- it might seem of guilding the lily perhaps, but certainly it has had impact on clinical transfusion medicine.

One thing that is increasingly discussed among many different groups are where genotyping tests are improvement on the serological tests, and I am pretty sure that you have had some discussion about that already. That is particular in the area of the RHD with the identification of weak D alleles and also the identification of D^{el} alleles,

and this can have clinical application in patients for transfusion and also of course in pregnancy. So this what we were sort of looking at with our particular cells, how could we improve them.

(Slide.)

So materials and methods of the study was that we simply isolated genomic DNA from frozen red cell samples from our in-house panel; and like many places prepare their in-house panels, we draw whole units from a donor, freeze aliquots in glycerol. And this generally --- are not --- repeated so we can very readily isolate genomic DNA from these samples.

The phenotypes have been deemed most likely based on their serological results, which I think is how we have all done it for long, and we tested 52 samples. We selected 52 samples for RHD zygosity, and the distribution is shown in red. We looked primarily at the R_1R_1. We tested 24 of those samples, 14 R_2R_2 and so on. These are not all panel cells included in our primary panels, but it was really a list of all the cells we had listed as test cells. We selected 59 samples for FY analysis, 33 samples that are currently doubled-dose Duffy Fy^a and 26 samples are apparently double-dose Fy^b. Then later in our studies we

selected 75 samples for DOA and DOB analysis.

(Slide.)

The next slide gives a very simplistic representation of the Pst1 RFLP analysis that we used in our lab, and this is the assay of Wagner and Flegel that was describe in Blood in 2000. Despite all its known limitations now, we have found it very useful as a screening assay, and in this assay we look for the digestion. We amplify fragments of DNA that covers the junction of the hybrid Rhesus, the five-prime Rhesus box and the three-prime Rhesus box that occurs in persons lacking an RHD gene. This is then digested with Pst1.

(Slide.)

You will see on the next slide that is a figure from Wagner and Flegel's paper in which the amplicon is shown on the top is 1,888 base pairs and then the various digest from the PST enzyme that will give a different banding pattern. In the first, if you click first, you will see that the first three samples are in fact RHD negative samples of varying big C and big CE types; and you can see that there is a definitive pattern notably with a band, a definitive band at 564 base pairs, which is probably the clearest one in this particular group. One click further on

and you can see that this group are three samples that are heterozygous for RHD, and they have the characteristic 564 band is actually lighter in this, but quite distinguishable from the deletion types. Then one click further on and you can see that these homozygous samples lack completely that 564 band and therefore you get distinguishable from the others. So we use this assay as a screening assay for those samples in which we are interested in zygosity.

(Slide.)

My next slide shows the results of that assay with the 52 samples that we tested, and fortunately I have got animations on these. If you just want to do two clicks you can show that two of the R1R1 samples were in fact heterozygous for RHD and not homozygous as we had predicted from the red cell serology, and on of the R2R2 samples was also heterozygous for RHD. It carried R double prime gene. What was also interesting was that we included three RO samples in this study, which expected to be R^0r --- to a population which contains a lot of African-Americans where you perhaps expect them to be double dose. We expected ours to be R^0r, and in fact they were. So in this group we show that three panel samples or three reagent samples of 52 gave unexpected results based on their serology, and these are

cells that one of the R1R1 samples was a regular donor that we had included in our screening cells. So again, of potential clinical importance.

(Slide.)

On the next slide -- oh, this is just a summary. Sorry. This shows because we know from the studies of --- and --- and others that PstI RFLP analysis is not reliable for detection in non-white populations. We did send the samples to the Sanquin labs and --- in Rotterdam confirmed those by real-time PCR, so we were pretty that our results were what they said they were.

(Slide.)

The Duffy analysis we used an allele-specific approach to --- Fy^a and Fy^b stages as shown on this slide, and we incorporated primers ---. So we incorporate primers for the mutation GATA box and also primers for the mutations at the Fy^a and Fy^b determining nucleotides.

(Slide.)

So if you look at the next slide you can that for each sample that we run bring on high throughput of assay. Each sample that we run we test four PCR mixes, and so in the first --- you can see the primer panel below. So there is one, two, three, four. One primer, the first primer pair

is Fy^a with the GATA mutated. Two is Fy^b plus GATA mutated. Three is Fy^a plus normal, and four is Fy^b plus normal. But you can see in the first sample the only amplification --- primer pair three, and this is actually an Fy^a homozygous. With panel two both alleles three and four have amplicons, and that describes Fy^a Fy^b heterozygous. --- the first panel --- first panel. Panel three shows Fy^b homozygous, and this last panel shows amplification only with primer pair two, which indicates this person is an FY normal, Fy^0Fy^0. So there are many methods for determining --- genotyping. This is our method here.

(Slide.)

When we looked at our ASP we showed that of the 33 samples that we thought --- Fy^a three of those were in fact Fy^a Fy^x and surprisingly -- well, surprisingly in our population two out of the 26 samples that we thought we were Duffy -- sorry, double dose of --- were in fact Fy^b Fy^0. So five out of 49 our panel red cells were heterozygous for either Fy^a or Fy^b but we expected that they were in fact double dose. I think 8.5 percent represents quite a high number of either falsely determined or falsely called samples, so that is just something to think about.

(Slide.)

Two clicks on gives you the next slide, and this is just a very quick schematic representation of the background of the DOA and DOB polymorphism determined by --- at nucleotide 793; and we use the allele-specific PCR described by Wu et al in Vox Sanguinis 2001, and amplify at either 162 base pair amplification of DOA or 161 base pair in the case of DOB fragment, so it is very straightforward.

(Slide.)

On the next slide it shows the results of that testing. Of the 75 samples we determined their genotype shown there: 14 apparent DOA are homozygous, 39 DOA DOB heterozygous, and 22 DOB DOB are homozygous. I have given the incidence on the right-hand side, but this not a random sampling. These are our panel cells, and who knows how -- this is not just random donors. These are our cell panels. They had been selected for other reasons. However, the distribution is not too unlike you would expect from the European population. So it was quite nice, it was quite comforting in that way, but the distribution of --- normal.

(Slide.)

So our conclusions from this little study was that the analysis showed that three samples that we thought to be RHD homozygous were heterozygous. One our screening Fy^{a+b-}

samples was in fact Fy^a Fy^x and had been on the panel for a long time, and that is very similar I think to Marion and Christine's --- in the New York Blood Center. One in seven of our panel of our Asian red cell donors showed a discrepancy between the predicted phenotype and the genotype, and this of course is the essence of the discussion today I think. This in an important issue for quality assurance, particularly when it comes to screening cells. Maybe not so much to panel cells, but then one could argue that the work --- is probably worth the effort.

(Slide.)

So should molecular genotyping be used as part of reagent red cell QC or QA? We now know the molecular basis of 28 out of 29 blood group genes. We know the molecular basis of many of these blood group polymorphisms. In fact, some of the criteria that are demanded by our regulatory agencies can only be met by genotype analysis, so perhaps we have to think that way rather than from our traditional approach of, "Well, should we incorporate it because we can?" Well, maybe we should look at the rules and what is expected of us to begin with. Of course the argument that we used for a long time is that antisera are increasingly more difficult to find and the production cots are rising,

and with the introduction of the beadchip in the US and hopefully our blood chip here we are hoping that microarrays are going to be the way of the future. Of course then life will be different because the costs will come down and we can do so much more with one platform.

(Slide.)

The next slide just briefly lists that comprehensive -- as I have discussed, comprehensive profiling of test red cells will aid faster antibody identification. This is certainly true. I think once you have all the information in your test cells certainly for those patients where the antibody specificity -- where the antibody of patients' serum contain many specificities having a clear idea of what is in your panel cells makes it -- speeds up an investigation considerably and can decrease time delays in obtaining compatible blood; and, as mentioned briefly, high throughput platforms will permit this, will make genotyping affordable.

(Slide.)

So just to end on the people that did the work, because most of this work was done long before I got to Lund. I just put it with words right here. So you can see the Lund people are listed here: Annika, Asa, Elisabet,

Pia, and Martin. From Sanquin we would like to thank Martin Tax. Of course thanks to Marion Reid and Greg Halverson who provided Dombrock antisera, and some of the work was sponsored by the Swedish Research Council. So I hope I can participation in the discussion. I want to see how the technology will work. Thanks.

(Applause.)

MS. KOCHMAN: Did anybody have any questions for Jill?

MR. YAZER*: Mark Yazer, University of Pittsburgh. Jill, in spite of the fact that you had some cells on the panel that you thought were double dose DuffyA by really were only single dose, did you find that you were having any clinical consequences from this? Were you missing these antibodies?

DR. STORRY: Sheryl, that is blocking out. I would ask if you could summerize for me.

MS. KOCHMAN: Sure. It was Mark Yazer from University of Pittsburgh. Wanted to know if the fact that because you found one of your screening cells to actually be heterozygous for DuffyA if you had any clinical implications from that.

DR. STORRY: Not that we know of, but then, you

know, that is the next step that nobody really wants to take back, to look back. I mean, it is all very well saying, you know, this may have dramatic clinical implications, but I don't think we or anybody else has actually taken a look at cases that have resolved those particular reagent red cells and noticed whether we have missed anything. So no is the answer to that. We haven't done that work. We have changed. I mean, it did prompt us to change our reagent red cells from that screening panel, but that is about it so far.

MS. KOCHMAN: Anybody else?

(No response.)

MS. KOCHMAN: Well, thank you again, Jill, for being a good sport in all of this.

DR. STORRY: Well, it has been fun. So, you know, it has been fun. I am sorry about that, but has been quite -- I have quite enjoyed the drama, as usual.

MS. KOCHMAN: Okay. Well, thanks again.

DR. STORRY: Thanks. Bye.

MS. KOCHMAN: Bye.

(Phone call ended with Dr. Storry.)

MS. KOCHMAN: Well, unfortunately today we have gotten ourselves behind, probably partly my fault for

getting us started a little late. We are scheduled to have the break following Dr. Karina's talk, so if you could just be patient and listen to her talk. She is another representative from the New York Blood Center, and I would like to give a little background on why I asked her to make this presentation.

You have heard us mention a few times that the FDA has seen some fatality reports where anti-JKA or anti-JKB have been -- that have been missed have been implicated in the fatality. One of the questions that keeps coming up is isn't there something we can do to make these antibodies to detect. Can you make super Kidd cells somehow so that we aren't missing these, and I thought that she might have some interesting perspectives on is there something else we can be doing or should be doing to help us find some of the things we miss.

Applications of Blood Group Antigen Expression Systems for Antibody Detection and Identification

by Karina Yazdanbakhsh, PhD

DR. YAZDANBAKHSH: Thank you to the organizers for inviting me here. So as Sheryl said, the focus of my talk will be slightly different to the other talks given at this workshop. It won't be on molecular genotyping of a patient

and donor samples, but rather applying our knowledge of the molecular basis of blood group antigens in developing reagents that can be used for antibody detection and identification.

(Slide.)

So I don’t need to tell this audience about the definition of a blood group antigen. Suffice to say that these antigenic determinants result from a specific sequence of amino acids that are present in one protein or present in several proteins and/or from those sugar molecules on oligosaccharides that are attached to the red cell surface proteins and lipids.

(Slide.)

As has been said over and over again, the genes encoding the proteins that carry these blood antigens have been commonly sequenced for the most part, and we know their structures as presented on this schematic drawing. It can be single pass proteins like the Kel and the --- or multi-pass proteins such as the Duffy and the Kidd.

(Slide.)

It is not the blood group antigens that cause problems in transfusion medicine, rather the antibodies than can cause the immune haemolysis.

(Slide.)

And to insure safe blood transfusion we have the antigen antibody identification process in place. Current methods rely on using multiple red blood cells, which results in a large number of antigens, and we apply a complex matrix of techniques to identify the clinically significant antibodies in the patient sample.

(Slide.)

So again, current methods rely on panels of cells that have got a specific combination of antigens present on them. Again, not for this audience, but we apply different techniques and based on the reactivity panels in a patient's sample the medical technologist can identify a particular antibody that is present in the sample. In this case it is anti big E, pretty straightforward. However, things can get very hairy when there are a number of other, a number of antibodies that are present such as --- reactivity. All the cells are reactive, and so based just on this pattern you cannot distinguish or identify all the antibodies in this particular sample.

(Slide.)

So the goal of a number of investigators in the

field back in the early ‘90s including Marion Reid was can we have system where we express single antigens and --- of our knowledge of the molecular basis of the blood group antigens, and can we develop an object and automated system that can be used for antibody identification. The kind of assays we had in mind was using flow cytometry and some sort of solid base assay such as ELISA for antibody identification. Also since we do know these, the molecular basis of these blood groups, can we use them to make recombinant proteins that can simplify antibody identification processing for such studies as for absorption neutralization studies.

(Slide.)

So this is the basic idea. Here is the red cells, the expressed number of different blood group antigen carrying proteins; and wouldn’t it be great if we could just express a single blood group carrying protein in a given cell line and apply them onto some sort of a solid base assay. In this case it is just showing some sort of an ELISA. So every well will contain a specific line expressing a single blood group carrying protein, and then you come in with your patient sample, add it to these wells, and whatever lights up you can identify that that is your --

that is the particular antibody that is present. Also this doesn't have to be a cell line expressing recombinant proteins. It could be --- proteins expressed or if we know more and more about the antigenic determinants we can have --- in these wells. So we have single antigens in each well and then you can just identify the antigens, the underlying antibodies in the patient sample in this way.

(Slide.)

So expression systems are amenable for large-scale production of recombinant proteins right now. The bacterial and the yeast systems are really great. The labs for large-scale production at low cost have a few antigens of interest or your protein or interest requires post-translation or modification such as glycosylation, these two systems are not really the best to go with. Baculovirus allows some limited amount of glycosylation and actually has been used to express soluble forms of blood group carrying proteins. We focused on the mammalian expression system since they do allow glycosylation of the transfected proteins, and there are some cell lines --- that are easy to grow and some even sort of easy to transfect.

The things you need to consider are what kind of a cell line you want to use. Since our interest is red cell

antigens, so you want your protein of interest to be -- to look like the naive protein on the red cell membrane. One idea would be to transfect these genes in gene erythroid cell lines that are available, and they are available. However, you run into the problem that for antibody identification you may have some micron reactivity, and that is exactly what we found. So you may want to switch to another species such as mouse or --- cells. However, you have to keep your line if your protein requires glycosylation the mouse and the human are not exactly the same, so you may run into problems there. Also red blood cell lines currently that are available are not that easy to transect. There are some --- lines that are easier to transect, so there are a number of things that you have to keep in mind, and it is always a toss between what is more important and what you are trying to achieve here.

(Slide.)

So what I would like to do today is to show you a couple of examples of the systems that we have used. One is the erythroid expression system and we have used a mouse erythroleukemic cells. These are the MEL cells, and another system is the --- T cells. These are the human embryonic kidney cells. To be able to drive the expression of

transfected genes in the case of the MEL we have used this PEV vector which has got human betaglobin locus control region that confers high level expression of the heterologous genes. In the case of the HEK cells, we have used vectors that have the CMV-promoter. These are strong promoters that help to drive the expression of your target gene. --- made both membrane-bound forms of our blood group antigens as well as soluble forms, and in the case of the membrane-bound forms we have used flow cytometry as well as ELISA as the absorption studies to actually show that these can be used and potential used in the clinical lab as well as the soluble forms. We have done antibody neutralization studies.

(Slide.)

So first is the detection by flow cytometry Here is the KEL protein. What we have done is to express the wild type KEL protein in these MEL cells using that EV vector that I told you about. This a transfectant, stable transfectant expressing the wild type protein, and what we are looking at here is by flow cytometry whether the --- expresses the different --- antigens, KPB, JSB, little k. As you can see, the --- is indicative of the reactivity. This is with the red cell, antigen positive red cell, and

here is our transfectant being able to detect the anti-KPB JSP and the anti little k at levels that are comparable to the red cell. So actually we have gone, although they are not shown here, but also --- cell lines of the --- antigens to the JPB. Basically JPA, JPC, big J and JSA, so now we have a panel just like our panel of our red cells that we can basically use by flow cytometry to be able to detect the antibodies in a patient sample. We have done those studies.

(Slide.)

Here is the Duffy protein. It has got a pair of --- antigens, Fy^a and Fy^b, and we have transfected again. We have got transfectants, stable transfectants expressing the Fy^a and the Fy^b, and now our Fy^a expressing cell line can specifically detect anti-Fy^a but not the anti-Fy^b antibodies and vice versa. Here again we have a system where we can detect underlying antibodies in a patient's sample by flow cytometry using these cell lines.

(Slide.)

We have also shown that these transfectants can be detected by ELISA, and this just an example of the kind of readings you can get. So the cell lines have been immobilized on 96 plates and then you come in with the patient sample, and in this case what I am showing is a wild

type --- transfectant and the big K transfectant expressing the big K antigen. Basically this the average of the OD values system by ELISA, so it is a columetric assay. You get a reading and it is an average of triplicates where the standard deviation is within 10 percent of each other from well to well and, you know, subtracting the background. These are the kind of numbers you get and then the ratio. Based on the ratio of these values you can make the conclusion that the example has anti big K. Again, as I say, we have done them for a number of transfectants that we have produced in the lab.

(Slide.)

So absorption studies are done in the blood bank to separate mixtures of antibodies, and they aid in the antibody identification process. So red cells that express the specific antigen are incubated with the test serum and the antibodies are absorbed the antigen. However, we have little mutations because red cells carry many antigens and you need multiple rounds of absorptions to be able to phenotypically -- are needed using phenotypically distinct red cells, which could be rare and in short supply.

(Slide.)

So the idea was can we use our transfectants that

are expressing single blood carrying proteins for absorption studies, and this is just to show again here is our wild type KEL protein, transfectant --- in our MEL cell lines. What we have here is this particular cell line has completely absorbed that antibody. These are the titers here of the anti little k, Kp^b, Js^b, and the parental cell line does not, which is just a nice control. Obviously the spectrum doesn't express the lower incidence antigen and is therefore not capable of absorbing --- anti Kp^a. Again here we have our --- tranfectants. They absorb --- specific antibodies in a given sera.

(Slide.)

So neutralization studies are another technique that is used to help in antibody amplification process, and it is specifically used to remove antibodies from antibody mixtures. Basically these inhibition studies are using fluids containing specific soluble blood group antigens. However, there is only a limited number of soluble antigenic substances, which they may dilute our test sera.

(Slide.)

Neutralization studies are usually directed at removing clinically insignificant antibodies, and one of the notoriously difficult antibodies are those against the ---

group antigen. These antigens are carried on this protein --- receptor one, and JoAnn --- and her colleagues nicely showed that recombinant protein --- of this --- receptor one can inhibit and neutralize antibodies in patient serum. So we actually repeated those studies, expressed soluble --- one. In our system we have used a vector that allows the proteins to be tagged so then we can easily purify the proteins and detect them just for quantitation. We have also expressed the different portions of this --- protein, and this a way when we did started these studies. We didn't know where the antigenic determinants ---, so this was a way of doing --- mapping studies.

(Slide.)

So this is just an example of inhibition studies on patient sera containing one of these anti --- antibodies. A couple of examples of inhibition. Again, soluble --- one can inhibit these two antibodies, so does one of the fragments. This is this long --- repeat D, but not the others, and here is a nice control with anti Yt^a where we don't see any inhibitions, just the control.

(Slide.)

So basically what I have shown today is basically we have expressed several both clinically relevant as well

as clinically insignificant blood group antigens at levels comparable to red cells, and these allowed us detect alloantibodies in patient and donor serum by flow cytometry as well as ELISA, and these allow -- they potential for automation, these two techniques. I have also shown that you can do absorption and neutralization of alloantibodies using these recombinant proteins, which will help in antibody detection and identification processes. They will simplify those processes.

(Slide.)

But a word of caution. These are all feasibility studies. We need a lot of work ahead of us. Basically one problem with these cell lines are that after freezing and storing of these expressed cell lines they lose expression. If you keep them a long time in culture they lose expression. So we really need to come up with improve systems, and they are out there to improve -- that would allow stably expressed cell lines in the sense that it is going to be there. It doesn't matter if you freeze them, thaw them.

It is really important to understand expression requirements for blood group antigens. What we have had problems with is trying to express the RH proteins at levels

comparable to red cells. We have been able express the RH antigen D just by using the Rh50 body in the 293 cell line, as well I was talking to Connie. She has expressed them in ---. However to get levels that are comparable to red cells we need to understand what are the requirements there. As I said also, there are a number of groups that also have expressed the --- 562 cell lines, but however the problem there is that, as I said, we have a lot of background using K562 cell lines for antibody detection we wanted to. So that is a problem there.

If we want automation we need to do epitope mapping studies. Really, you know, identify like epitope --- level what are these antigens so that we can apply them in some sort of a solid base assay. So epitope mapping studies are required, and that is it. Thank you.

(Applause.)

MS. KOCHMAN: As I mentioned before, we are running a bit behind today; s does anybody object to just taking a 15-minute break instead of a 30-minute break to try to catch up a little? Okay. So if you could be back in 15 minutes. Thanks.

(A break was taken at 10:23 a.m.)

MS. KOCHMAN: So we are going to move on to yet

another slight shift in gears maybe. We have Sandra Nance from the American Red Cross. She is the Director of the Immunohematology Reference Laboratories and I have learned is also an adjunct faculty at University of Pennsylvania. So we will have Sandra talk to us.

Donor Genotyping

by Sandra J. Nance, MS, MT(ASCP)SBB

DR. NANCE: Okay. So to begin, this is a topic that I am not too familiar with as far as some of the things that I am going to be talking about, but Sheryl asked me to try and cover it, so I will. I just want us to remember that molecular is just a different method as it goes through --- genotyping with an existing sort of test name, a result antigen typing, and with the possible exception of the --- of a testing of single embryo which might be considered differently, and I will cover that at the middle of the talk.

(Slide.)

So Sheryl very nicely gave me some things to cover, and more questions than answered. Will cost provide widespread adoption? What about DNA storage and security? And is one test enough, or will repeat testing be required? I guess I know a lot about the one and the three, but not a

whole lot about two, so I had to do a little bit of research.

(Slide.)

I did take a number of quotes that I found in the literature because I thought they represented things that you might want to think about, and I hope to instead of talking science here I hope to really just kind of open your mind about operational aspects and perhaps some other things you might have been wrestling with through our discussions of yesterday and today. So the transfusion medicine specialist of the future may have their disposal molecular techniques to detect red cell genotypes.

(Slide.)

Under the category will cost prevent widespread adoption, I looked at the "as is" situation and I am doing a lot of project management these days, so I always look at the as-is and then look at the "to be" and find out how you are going to get there. So the high cost of labor for testing and labeling in a fading labor market is familiar to all of us.

Many of the labs do perform two types on new donors and compare. If you have a repeat donor you may compare that with antigen typing on previous donations, thus

only doing one repeat test. We do know that Rh testing on the ProVue or Galileo may be automated for those facilities who have it, and that could be a test of record, but we do know that all other specificities and methods at least in the US are manual for the test of record.

We also have the ability to have automated prescreening methods with the PK7200 and the ProVue from Ortho, which limits the amount of manual screening need to identify antigen-negative donors. Thus you will be --- a very productive manual testing technique. We also have talked about the limited availability of licensed antisera. Keep in our minds about lows and highs, which are not really commercially available, and we have as the "as is" perform minimal molecular matching for patients with complex serology and inherited mutations.

(Slide.)

What is the labor situation? Well, I have some data from the Red Cross I thought I would share. We have approximately 300 budgeted FTEs across the US in our 36 regional locations. We have approximately a 10 percent vacancy rate, so that means minus 30 people across the whole system. We do know that time to hire is variable in many different labor markets, but one to six months was the

average; and this was one point aspect in time in March of '06, so really just a point in time. Time to train is three to six months, but to get fully experienced in the IRL most folks would view that as two years.

(Slide.)

I wanted to tell you a little bit more information about the American Rare Donor Program, seeing how molecular typing could really help us out there. In looking at our 35,000 active donors in the American Rare Donor Program, about 90 percent of them phenotype rare, which means that they are negative for one antigen in each of five systems. Under 10 percent are high frequency antigen-negative rare, so obviously that is a place we need to work on; and IgAs are just barely a line here, so there are very few of those. So there is a lot of opportunities here I think.

(Slide.)

Keeping in mind that, you know, we may talk about localizing this molecular testing in different centers and maybe few and maybe a lot, but I wanted to point out that, while these contribute many of the high prevalence antigen-negative rares to the American Rare Donor Program, these are extremely important numbers over at the side as well. So everyone that contributes these high prevalence antigen-

negatives are really important to us.

(Slide.)

To show you some data and why I made the comment I did yesterday, data from 2004, ‘4, and ‘5. The total requests that we have gotten into the system, the percent that are filled, really not much of a change here. The number of partially filled means that they asked for four and maybe got two, and we have made a change in that which I am really happy about. However, the percent not filled, and this represents actual patients not getting the blood they need, half of which probably die. This number needs to be zero, so I am really looking for some energy here in what molecular testing may bring to us in that area.

In looking at just data for the last year and a half starting in January of 2005 to June of 2006, I separated out the sickle cell disease patients. So this is a subset of this number here, and just to point out that I don’t think that there are much differences between these percentage numbers as far as whether it is filled, partially filled, or not filled, but we do need to keep our eye on that.

(Slide.)

We do not fill of the requests that we get of

course, as you know. So these are the imports for the American Red Donor Program in the last two years, 2004, 2005, that we got from other countries and just to look at the phenotypes to show you what we need.

(Slide.)

So will costs prevent widespread adoption? Well, the "to be" -- which is we looked at the "as is" and now we are looking at the "to be" -- I think we are going to still see continued shrinking of the labor availability. We may have some light at the end of the tunnel that is not an oncoming train with phage display technology, and Dr. Siegel will talk about that later. We will have availability of some semi-automated molecular testing. I think that will help us. We may have the concomitant activity of increased expense for reagents, and instruments and supplies remains to be seen for us in the US, but it does expand our molecular matching possibilities, and electronic antigen-negative labeling will be coming which will decrease the time resources for testing and for labeling.

(Slide.)

It does remain to be seen the cost of the semi-automated molecular testing. Although appropriate pricing might be equal to the current resource that we expend with

our manual tests for limited donor testing, so I am looking forward to that. I think it is a given that it won't be all donors collected unless we are able to have a very inexpensive or we are able to do a single test event on each donor. It means that we don't repeat them on each next donation, and widespread donor testing can likely only be achieved with totally manual methods if we get some funding. I don't see that to be widespread donor testing with molecular methods based on the resource that is needed.

(Slide.)

So I designed a little possible workflow. It is the opposite of the approach we heard yesterday, but you may choose whichever you want. What I took was a repeat donor from the South African experience and that they find that it is much more cost efficient to start testing for serology antigen-negative blood with repeat donors because there is a certain number of fallout rate from first-time donors.

So in the event that the serologic antigen typing is known, and here we open up ourselves for look-back. So if it is yes and it is African American -- I divided it between African American and not African American. If it is African American we may want to do DNA looking at the Rh and highs. If it is not African American we might want to do

the commons. Here is where we open ourselves up for a look back if the result of DNA may be different that the serologic type we already know. Then my thought is that at the time of the event of the molecular testing on that donation we do a serologic type for negatives and resolve the issues, and the same on the other side.

It is a little bit happier for us in the Blood Center of course is the serologic type is not known because we don't have look-back opportunities. So we would do DNA on the commons, if it was African American we would DNA, do Rh and highs, and then serologically type the negatives. So that is just my operational look at a potential workflow that we might find achievable.

(Slide.)

David Anstee made a great remark in one of his reviews for Transfusion that I wanted to share with you. Molecular typing for ABO and Rh is not a simple matter of identifying one or two SNPs. We talked about that. The genetic basis of both antigen systems is complex and will require the careful design of multiple reactions before a bullet-proof molecular tying system suitable for all racial groups is achieved. So we could like have combined all two hours that we talked about that into this little slide.

(Slide.)

Okay. Here is what I don't know about. So what about DNA storage and security, and I returned to the web of course to try to help me with this. But the things I thought about were ethical concerns, and my thoughts are that antigen typing info is the same as serology. So I don't think we need to be so concerned about that. Storing DNA I think people fear it is going to be used for other things. Potential for distribution for samples of interest and/or proficiencies may be something else we need to think about, and then notification of donor if it is important to the health or genetic planning. The things I thought might fall under there are hemoglobin S and if we HPA 1A screening and then we do an antibody test on the women then that might also be something that would be important to the health or genetic planning.

(Slide.)

So borrowing from some of our friends in HLA, there is something on inspector checklist with regard to storage of the DNA. Just to insure that the samples are stored under conditions that preserve the integrity of the sample for the things that will be tested, and the inspectors are actually supposed to test for -- I mean check

for written criteria for short-term and long-term storage of DNA.

(Slide.)

In looking at another review that was in a journal by K. Smith who was a diplomat of philosophy and religion, access to genetic testing should be treated the same way as access to new medical procedures and medications. Namely withheld from the general public until proven safe and effective in larger-scale trials, and I think that is the direction we are going.

(Slide.)

There was a taskforce on genetic testing and there was definitions of that, and it is here for you to read. I think that probably the last part is the most interesting. Such purposes include predicting risk of disease, identifying carriers, establishing prenatal, newborn, and carrier screening, as well as testing high-risk families and individuals. So that is what was determined to be the definition of genetic testing.

(Slide.)

So there are three categories that seem to be defined in the literature. Diagnostic, presymptomatic -- and that is where I think this might come in perhaps -- and

reproductive decisions. Hemoglobin S, HPA 1A and the D antigen might fall into those.

(Slide.)

So in scope for us at least under consideration, and I would say this is the patient side, not necessarily donor side, really looking at the patients. For hemoglobin S, paternal testing, and prenatal screening; and I think out of scope, obviously subject to other people's opinions, donor antigen testing.

(Slide.)

In another journal looking mostly at breast cancers, but I thought it was interesting, the DNA test should safeguard the welfare of the person being tested.

(Slide.)

On the Council of Europe, and this was the latest reference I could find, so I bring it to your attention. It is from 1996, 10 years ago. DNA testing may be performed only for health purposes and subjected to appropriate genetic counseling. So I might look to our European colleagues to see if that is still current or they have heard of that.

(Slide.)

Then from the US Congress, genetic testing was

defined as the use of specific assays to determine the genetic status of individuals already suspected to be at high risk -- so that knocks out all the donors -- of a particular inherited condition. The term is genetic test, genetic assay, genetic analysis. They are all used interchangeably to mean the actual laboratory examination of samples.

(Slide.)

Genetic screening, which is different from the first, the slide just before, uses the same assays employed for genetic testing, but is distinguished from genetic testing by its target population. So any of our things that might possibly fall in probably fall in here.

(Slide.)

So what are the thoughts about DNA storage and security? There needs to be some standard development on the rules of engagement here I think. Storage of patient and donor samples may be somewhat different. Certainly we should unlink the samples for interest only. We shouldn't be sending samples around with patients' names on them, and I would assume that we would always unlink proficiency samples.

(Slide.)

Next topic. Thank God I am done with that one. Is one test enough, or will repeat testing be required? Great question, and I think it hinges on FDA approval of automated platforms and keeping in mind that new discoveries of mutations would have to be then incorporated into the platform, and then accuracy of testing. Proficiency standards, and we are going to have some discussion about standards of detection.

(Slide.)

In another article that I drew some information form, and it has nothing to do with blood grouping, but looking at microarray platforms in other fields. I think that we will have some differences between the European and US platforms in that the measure their expression of the same gene with different precision on a different scale with a different dynamic range. So this has already been known within the microarray field for a while, and if you do look at the two together, which we might do as, you know, blood banks would do, it might become compromised when applied to data generated by another platform. So maybe the two aren't the same; maybe it is a little bit more like coagulation assays where each different instrument has a different range and a different proficiency value.

(Slide.)

So why test more than once? Well, I think that we have concerns over accuracy, so that would definitely need to be in consideration, realizing that the techniques we have aren't perfect either. I think we have a concern that serology is different than molecular, so we may need to marry the two, at least for awhile until we get to the "to be" that is, as Dr. Seigel said, 20 to 30 years from now. We do have a lot of concern over the identification of the specimen if it is not totally automated. People make mistakes. We need to have --- ID throughout the whole course of the test if we want to do one test, and I think concern over changes and new discoveries.

(Slide.)

So my thoughts are about those things: So we would need a validated method. If we have a concern we might want to type once with each method per donor if we are able to have --- ID, and if we don't have a totally automated system we are going to need to type twice. We are probably going to need to type each donation I think. We need to talk about that a bit, and then new discoveries. We might have to change the platform with each new discovery. I did want to point out, thanks to Marion Reid for bringing

this up to me, that we do have an SOP for the American Rare Donor Program which applies to accredited labs by the AABB and ARC. So the American Rare Donor Program SOP allows molecular assay results to be used as historic data. Not for labeling the product, but for informing them. Especially with Dombrock it is has been extremely helpful.

(Slide.)

So what about genomic DNA standards? I think we are going to talk about that later. One of the articles that I brought up did show a fundamental problem in some of the things with microarray analysis which had to do with lack of common standards, spotting efficiency, labeling efficiency, transcript representation, and hybridization. So I think those are things that are probably common to the field that we need to apply as we look for blood group.

(Slide.)

In another article out of NIH, calibration standards need to be stable over time, homogenous, withstand shipping and normal storage, and actually contain a reasonable amount of DNA which would be useful. There is not as I know a standard for blood grouping, but there is a standard which regards DNA, so the laboratory shall check its DNA procedures originally or when changes are made to

the protocols against appropriate and available NIST standard reference material or standard traceable to an NIST standard. So hopefully we will be looking to develop that in the future.

(Slide.)

So is one test enough or will repeat testing be required? If the molecular type is wrong is it then, as I alluded to yesterday, a limitation of the technique like with the Olympus PK7200, or is it a recall if you type it wrong? Some of the things you already know about.

(Slide.)

The last thing I would like to leave you with is another quote from David Anstee which I think summarizes all of our thoughts. One can envision kits suitable for molecular typing of individual patients being used at the blood bank and electronic interrogation of the blood center database for selection of the most suitable donations available. So he said that last year.

So in conclusion, I think we ought to think about patient testing maybe in the realm of genetic testing. If it is done under physician order I think that we are probably still covered for the interpretation and the counseling of the patient. I think the rest are potentially

genetic screening, and it is no different than the techniques we use right now that yield the same interpretations. Thank you.

(Applause.)

MS. KOCHMAN: Now we are going to move to Dr. Dan Bellissimo who is going to talk to us about proficiency testing. He comes from the Blood Center of Wisconsin.

Proficiency Testing for Molecular Assays

by Daniel B. Bellissimo, PhD, FACMG

DR. BELLISSIMO: Okay. I am going to be talking about proficiency testing for molecular assays, and I do work on the College of American Pathologists Biochemical and Molecular Resource Committee. That is a committee that puts together proficiency surveys for molecular genetic testing, and I also work in the QA Lab Practice Committee in the American College of Medical Genetics, so my comments are likely to reflect the work of those groups.

(Slide.)

First of all, I just wanted to make everyone aware that there are multiple guidelines available for molecular genetics, and I think a lot of these standards and practices would apply to DNA testing regardless of where they occur. First of all, there is CLIA. It is not completely strong on

the aspect of genetic testing, but does contain a number of guidelines about performance validation for assay development.

Then there is also the American College of Medical Genetics Standards and Guidelines. These guidelines cover areas --- genetics, biochemical genetics, and molecular genetics. They deal a lot with the different kinds of problems people see in molecular type testing, and a lot of them are directed at problems that have been seen over the years in molecular testing.

There are also multiple guidelines from the National Committee on Clinical Lab Standards. There are standards both for molecular genetic testing, DNA sequencing and molecular pathology. The state of New York also has laboratory standards in regards to molecular testing.

Then there is also the CAP, College of American Pathologists Checklist for Molecular Pathology Labs. This actually a checklist that is used to inspect your laboratory if you are going to be CAP certified, which is the gold standard in laboratories performing molecular testing. These checklist items are very much directed at techniques and the things required to have quality testing in molecular pathology laboratories.

Finally there is the American College of Medical Genetics Disease-Specific Guidelines, and these are guidelines that are written for specific disease diseases where there are specific problems occurring in the testing community where the testing is complicated and requires specific professional direction to insure quality testing. I think from Marion Reid's comment this morning CBBG has recognized the need for such kind of guidelines in the area of blood group testing also.

So these as a whole, these generate kind of the standard of practice. I said, they have a lot of different comments on techniques and controls necessary for techniques. Those are especially true in the ACMG Disease-Specific Guidelines where for example in the CF guidelines they go through the multitude of different assays platforms being used, what type of controls and things should be considered for each type of assay technique.

I will also mention those that the guidelines in regard to microarrays are probably at an early stage. There is a lot in development going on just because of an assay like cystic fibrosis which run a large screening panel, so a lot of guidelines are being developed, and I will discuss some of those later.

(Slide.)

I also wanted to make everyone aware that the ACMG also has guidelines for prenatal molecular genetic testing, and I think a lot of this again applies to what happens in HDN testing. These are just a summary of some of those guidelines. That the mutation status of one or both parents as appropriate be tested in prenatal testing. I think that is a concern in red cell testing where there are a number of varying alleles and it is particularly important to make sure you test the mother to make she doesn't have a false-positive Rh variant before testing the fetus.

That laboratories should have some kind of followup program to try to monitor the accuracy of their prenatal testing. That is sometimes difficult to do. That laboratories need to find some way to make sure they are doing accurate diagnosis.

The last two comments relate to the problem with maternal cell contamination that can occur in prenatal samples. The basis of these recommendations is that laboratories need to understand how maternal cell contamination would affect their prenatal result. So labs should have methods for assessing the presence and amount of maternal cell contamination. These are typically by using

VNTRs and SCRs to do chimeras* analysis, and that the methods should detect the levels of maternal cell contamination that would lead to a diagnostic error. This is typically done using DNA mixing studies, and I just wanted to illustrate that this is important both to consider for the type of technique being used and also the type of mutation that you are trying to detect.

(Slide.)

So this is an example of a --- muscular dystrophy assay, and most of the mutations in this disorder are deletions, and it is a deletion on the X chromosome. What I am showing here is a multiplex test which tests for a number of different exons in the dystophin* gene. In this --- column here is zero percent or is an affected male, and I think you can just see that these -- well, for simplicity just look that that these top two bands are missing and this patient was deleted. So what we do is then dilute that sample with a female's DNA mimicking a contamination that might occur in a prenatal sample, and the question is at what level does contamination confound your diagnostic result.

For those who have good eyes can see that around five percent contamination with maternal DNA in this test

leads you to detect these two bands from the maternal normal chromosome. It could potentially lead you to an incorrect prenatal result. So this would tell you if you were doing such a deletion test that you need very sensitive methods to detect maternal cell contamination, because things below around five percent or below could confound your result, so that would require very sensitive techniques.

(Slide.)

Then I just want to contrast that with another method in another application, and this is a test that mimics contamination in a prenatal KEL genotyping assay. This is an allele-specific technique which is going to differ because there are two reactions per sample. The first one detects the K1, and there is a K1 homozygote and the K2 reaction is negative, and here is K2 homozygote. The K1 reaction is negative and the K2 is positive.

But in this assay there is a PCR reaction specific to detect the paternal allele that the mother does not have. So what was done in this assay was to take a sample that was heterozygous mimicking a heterozygous fetal and then just do serial dilutions out with maternal DNA. That would be K2K2, and ask the question, well, where do we lose the detection of that paternal K1 allele. Actually at one in 64 this band

is still clearly evidence, and if you will look closely at one in 128 you can still pick up this K1 band. So these assays are very insensitive to maternal cell contamination, and we have seen a number of prenatal samples that we know through other testing that they are 95 percent maternal DNA and that we are still able to detect the paternal allele.

So that just contrasts the difference between different methods and different mutation types, but it is under the obligation of the laboratory to understand what level of contamination would affect their assay. Laboratories that don't have the ability to detect contamination would have to send these out if the laboratory did not have -- you know, if the fetus typed the same as the mother and they couldn't rule out that possibility.

(Slide.)

So I am going to talk a little bit about the ACMG/CAP proficiency testing program. So this is a program that helps to assure good laboratory performance, and the proficiency survey is just one part of that. The CAP MLG survey is the molecular genetic survey. It includes a number of different genetic disorders, but also includes the RHD gene. The purpose of that program then is to, first of all, assure laboratory performance, but to also look at the

problems that we see and the results there. See if they are method based, see what kind of interpretive problems are, and then write participant surveys to help laboratories fix those problems. So it is very much trying not to be a punitive thing, but an educational thing to help laboratory performance.

Also to help that laboratory performance as we see, complications in the proficiency testing. That is sort of what triggers the AMC to start thinking about whether disease-specific guidelines are needed in a certain test. If there is a prevalent error occurring in different assay methods, and they have developed these for cystic fibrosis and --- and Huntington's diseases because of this.

The final part of that program then is laboratory inspections. I mentioned the CAP laboratory inspection checklist which is used to inspect laboratories, but the CAP and ACMG have done a lot of work to make sure people inspecting molecular laboratories are experts in molecular and they have the ability to see if there are problems there. Some of it is getting the data from the proficiency surveys. If the committees are seeing a problem with proficiency that this information gets back down to our inspection crews that go in so they can look at problems

there and try to figure out the problem the laboratory is having.

(Slide.)

So proficiency testing programs ought to assess at least three different components of proficiency. One is the preanalytical, and this is the receipt and processing, and I think we have heard people talking about proficiency surveys and seeing clerical results. In some ways we are kind of glad they are not analytical results, but clerical results are also a problem if it may lead to an incorrect result. People are supposed to treat proficiency samples just the same as they do other laboratory samples, but I am sure most people handle them with the utmost care to make sure there are not errors in those. They even do more than they probably do in regular samples. So the fact that there still errors in handling of these samples is concerning.

The other problem is in processing of the sample, which is a very important part of molecular testing. Especially, you know, we are talking about lots of kits being available to purify DNA. I think the impression is I just have to throw blood on this kit and I will get something good out the other end and I am going forward. I think those of use who work with DNA a lot know that it is

not necessarily the case, especially in the type of patients, clinical patients that come in who have had transplants and multiple transfusions, that these samples are not normal blood samples. I expect that we will see that in microarrays, that the quality of DNA going in is of the utmost importance to the performance of the chip.

But it s a problem in molecular testing of how to provide sample. Ideally you would provide the exact same sample coming into the laboratory, as a blood, but for rare genetic disorders this is almost impossible to do. So what we have done is built up a good resource of control cell lines, and DNA is provided is the laboratory, and it is the highest quality DNA that we can provide to make sure people do not have problems with quality DNA as they do the proficiency testing. But ideally it would be a blood sample or whatever sample the laboratory would be analyzing in that test.

The next part is the analytical result. This is just the test result, and I think most of the time people spend most of their time making sure their analytical results are correct. Usually this not as much of a problem in a robust assay format, but certainly there can be analytical problems in certain test methods, especially with

variants and polymorphisms that may upset the detection of specific mutations that we are trying to detect.

The last part is the post-analytical, and this is the interpretation reporting. This part has become a lot more important in proficiency testing and our committee has spent a lot more time working on it, because I think in the diagnostic kit assay world what we have happening is a lot of the tests that have become big send-out tests like cystic fibrosis and there are kits available what we find is people are able to a lot of times get an analytical result, but they are not able to accurately interpret the result and what that means. So we have actually started grading both the analytical and the interpretive component of these proficiency surveys, because it does little good to get the correct the analytical result and then interpret it incorrectly which still leads to an incorrect clinical action. So that is a very important aspect, especially in complicated testings that not only can people get the result, but they know what it means. I think what we see, again using the cystic fibrosis as an example, which is a complicated genetic test, that we see a number of laboratories offering this because they are able to get a kit, but some of the complicated genetics involves kind of

confounds in the interpretive part of the test.

The other important part of this program then is the is the participant summary that is written each proficiency cycle, which is twice a year, which we try to summarize the problems we have seen. Whether any, you know, method-based or sample switch-based, and if there are any suggestions or recommendations in regard to that performance. Many times, as I said, we see specific problems in the surveys, and that leads to a generation of developing guidelines and everything to help laboratories and us redoing our surveys to try to get at that component of the laboratory error.

(Slide.)

So what are some important resources for a proficiency testing program, and I think people have brought these up also for red cell antigens. The most important part is well-characterized quality control materials, of which we have been better at assembling in genetics. It is really important to be able to have materials that you can send out to laboratories to do these proficiency surveys. We also use laboratories to QC our materials. The materials go through quite a bit of testing to makes sure they are what we think they are before they go out. So when a new

cell line or DNA sample is put into use in this survey it gets tested by two independent laboratories.

We call this pretesting, to make sure that the mutations are what they are before they get sent out on surveys; and also the company that produces these cell lines and DNAs, when the prepared a new lot to go out for proficiency survey that sample again is sent out to laboratories to test to confirm that that mutation is as it should be. These were put in place because of past errors in production of some of these lines and different laboratories receiving different materials. So there is a lot of different testing going on to make sure these materials are correct.

Finally, you need experts to select these samples, review the data, assess problems, and write participant summaries. Of course it requires lots of administrative support to get this all done.

(Slide.)

But I do want to emphasize the importance of quality control materials. They are important not only for proficiency testing, but for quality control and test development validation. A lot of times what will come out of recommendations, and I am sure the same will be true

here, is that lots of different variants you have to worry about and you have to understand how your test method would react each of those variants. That can only be done if people have access to the type of variants and controls they need to do those tests.

(Slide.)

So what are we doing for quality control materials in genetics right now? A lot of these are just laboratory samples that a lot of them are used. We take samples that are unusual and blind them for controls in the future. We use them to send out and do sample exchanges. There is also a big stored -- a big set of cell line controls at the Coriell Cell Respository, and within that one of their sets of cell lines are called a human genetic cell repository and they contain a number of different materials with different genetic mutations. You can go to that site and search for different things. You do have to be careful in knowing which of these materials have been qualified or not. A lot samples are sent into them, and they immortalize them and make cell lines. Part of the process that is going on with those is that laboratories are qualifying those materials so you can be assured again that the mutations present there are what you think they are.

(Slide.)

The other part of that is something that is called the GTQC, which is a Genetic Testing Quality Materials Program. This was a program done by a number of different people, but Lisa Kalman at the CDC was a coordinator of it. The idea was to help the genetic test communities obtain appropriate and qualified QC materials and to facilitate any kind of information exchange that had to go on between the people to get these materials, which essentially was a patient sample into a laboratory that would immortalize it and then develop cell lines, and to coordinate all the -- you know, so coordinating all those efforts to collect and distribute and test. A lot of testing goes into these materials so they are qualified for use in testing. They have done a really good job --- so far putting together available materials.

(Slide.)

I just wanted to point out what they did in each of these areas. Fragile X and Huntington's disease are two disease with are caused by tri-nucleotide repeat disorders, so the diagnosis of a disease depends on the size of this repeat within these people's genes. So it very important to have accurate sizing of these genes in order to give the

correct clinical result; and what the GTQC did was find samples that were right at the borderline of people being uneffected and effected, so these would be great controls to be in molecular assays so that the laboratory could assure that what they were testing and measuring against these standards. So they have put together some great size standards for those two disorders.

For cystic fibrosis they pulled together a lot of different rare mutations that are in the panels now for testing, and a lot of those again were very hard to come by. Certain individuals have them. Now they are all available.

They have put together one of the new realms of microarrays that will be coming out, are panels to test for common mutations in the Ashkenazi Jewish population. There are certain disorders that are more common there, and again there was a set of control materials needed so everyone could quality control their assays, and they have also put together pharmacogenomic markers. As I said, I just see this as a program that is growing.

(Slide.)

I think the need for controls in the blood group system is much similar to what is going on here, especially as we move to chips. Those types of control materials can

be immortalized cell lines, DNA. I mean, there is also possibilities of even this whole genome amplification, clone controls, and then also synthetic controls which I will mention a little bit more about in a second.

(Slide.)

So controls for a multiplex or chip-based assay I think are a little bit different. Genomic controls are not ideal in this multiplex testing mainly because it is very difficult to use a genomic control for every different polymorphism that would be on a chip. Our recommendation right now in the cystic fibrosis assay in that regard is to rotate the genomic control so each assay we are running a different genomic control, different mutation control through the assay because it really is not possible to run them all at once.

As this complexity continues to increase I think synthetic controls are going to become more important tools for our use, and I just -- these are just some of the synthetic controls that are available for cystic fibrosis. If you are not aware of why this test has become so common in the United States, it had to do with the recommendation made a few years ago by the American College of Medical Genetics and the American College of Obstetrics that women

of childbearing age or are pregnant be offered testing for cystic fibrosis. So this lead to huge increases in the screening for mutations. The ACMG came out with a recommendation that, right now we have got 23 different mutations, the most common mutations be tested in the population, but actual test platforms go all the way up to 70 to 90 mutations that different people are testing.

So people have started to try to work on synthetic controls to help control these kind of bead array platforms. One is made by the Maine Molecular Quality Control, Incorporated. I will show you a picture of what they did in just a second. It is interesting because it is a synthetic DNA that contains 38 CF mutations, and they have suspended it in a blood-like matrix. So it looks like a blood, but it is not, but it goes right through your extraction procedure just like a blood sample would. So again, a good property of a control, because your extraction process will be evaluated also when using this type of control. AcroMetrix also has a synthetic DNA that contains CF mutations, and then I will mention the synthetic oligonucleotide mixture that Sacred Heart Medical in Spokane came up with.

(Slide.)

So this is what Maine Molecular did to make a

cystic fibrosis control. They basically had this huge plasmid DNA of about 20,000 base pairs, and it has a backbone pretty much of the CF gene. The CF gene has 24 exons, and you can see all those 24 exons are present on this. What they have done is make a synthetic construct that each of these exons and the different mutations in cystic fibrosis are incorporated, and they have a couple of different of these plasmids that put different combinations of all the cystic fibrosis mutations in them. So this is the control that has been put in the blood matrix, and you extract it and test it. It is a way to create homozygotes, heterozygotes, and all different kinds of things by using these things in different combinations.

(Slide.)

The other method that is being out are oligonucleotide base controls, and so basically the way this works is you have a genomic DNA that has a mutation and you create a long synthetic oligonucleotide that spans this region of the mutation all around it. You tag it with two common primers, two common sequences, and this is you would do this for each mutation that you wanted. But they would all have these common sequences at the end, and then you could just take all these sequences and amplify them with

these two sets of primers and create more of them. So you creating very little, small DNA segments that all contains these little mutations.

This was a system developed by Dr. Bejjani at Washington State University. The difference between this control is because this is such a short region this would not contain the normal primer binding sites that you would use to amplify this DNA construct. It would be able to be put in your detection system for like an allele-specific extension to bind to your array, but it wouldn't be an amplification control. But that is the difference, but it would be a way of testing that all your different SNPs on the chips would be detected.

(Slide.)

So proficiency programs for red blood cell antigens right now, there is the CAP survey which includes RHD only so it is very limited. There is the ISBT workshop, and people have talked about the multiple antigen systems that were tested there. There was sample exchange going on, and as I said I have started this program with Marion Reid quite some time ago. I didn't realize that it had gone international, but that was basically sample exchange between laboratories, and our laboratory also uses our in-

house comparison to serology which gets done on all the blood samples.

(Slide.)

The CAP survey, there was very limited participation in this survey. There is only nine or 10 participants. The performance has been excellent, but we haven't done any challenges on variants. About half the laboratories in there are testing for the pseudo gene, and we need to identify better quality control materials in the RHD area to test people's performance on variants.

Expansion of the survey has been a little limited because the low participation makes it hard to break this out and expand it, but I have a feeling some of the limited participation is because this is grouped in with a bunch of genetic disorders, and I have a feeling most transfusion labs are not testing for fragile X and cystic fibrosis and all those kinds of things so don't want to pay for the survey just to do RHD. So that may be something that has to be looked at in the future.

(Slide.)

We have already talked a little bit about the ISBT workshop and what was seen there. I will just point out that it is important that the number of participants has

risen quite a bit from 2004 to 2006.

(Slide.)

In regards to performance, I think the first year had an error rate of about five percent, and it dropped to one percent in 2005. Christine talked about the results in 2006, which looked like it went up, but it was mainly due to a couple of laboratories. But I think the important part of that workshop was the recommendation that they made that said use of adequate controls and testing for variant alleles, and I think this just goes back to what we discussed as the importance of quality control materials being available and also standard practice guidelines. That basically I think covers all the types of issues that we are seeing in this workshop.

(Slide.)

So in summary, proficiency testing, it tests the ability to accurate determine and interpret a test result, and the quality control materials are really important for this proficiency test development and validation, but I don’t think they are widely available. I know groups have assembled samples, but it would be good to have some kind of collection fairly easily available, and I think something has to be put together to make that all happen. Finally,

the availability of a proficiency testing survey in the US is I think limited by the CAP survey and what Marion has put together. Okay. Thank you very much.

(Applause.)

MS. KOCHMAN: Our last presentation before lunch is Dr. Don Siegel from the University of Pennsylvania, and he -- we are really shifting gears here because we are going to talk about phage display technology and maybe getting back to serology instead of getting away from serology.

Overcoming Limitations in Current Pre-Transfusion Compatibility Testing Methods Using Phage Display

by Don L. Siegel, PhD, MD

DR. SIEGEL: Okay. Thanks very much for inviting me here. What I wanted to start out by saying is one of the issues that came up yesterday which I kind of raised is what we think blood banks will look like in the future, and a number of people have said that some aspects may still require serological technique. One of the questions that I have is not just what serology will continue to contribute, but what would the lab look like in terms of its technology. Would the serology still be done using agglutination? So you would have half of your lab would be a genetic lab, and

half would be a conventional serological lab? Or is there some way of combining the platforms together so that whether you are trying to detect genes in a person or proteins in a person with antibodies that the readout could still actually be combined together in the same kind of platform such as a microarray? So this seems kind of strange as to how a microarray could be involved in serology, but that is what I am going to try to get across this morning before lunch.

(Slide.)

So the outline of my talk is first I am going to just review some of the drawbacks of current pre-transfusion testing methods, and then I will give an overview very briefly of phage display and then how phage display can create conventional agglutination-based antibody reagents, and then how I think it can be used to create some novel what I can refer to as genetic-based antibody reagents.

(Slide.)

We weren't asked to do this as speakers, but I just want to mention that in the effort of full disclosure there is a company called Pheno Tech which was founded by the University of Pennsylvania, and many of the technologies that I will be talking about in my talk have been licensed by this company and I have an equity interest in this

company. So I just wanted to mention that.

(Slide.)

So if I put on my medical director hat for second, this is what has been going on at my hospital. This is if you look at the work in the blood bank as a function of the number of types and screens we do, this is from just a few years ago where it was about 30-something-thousand. We are up to 80,000 types and screens last year, and this is a reflection of increased surgery, many other types of programs that require a lot of blood like --- programs, --- programs, and of course the labs and the blood bank don't really receive any kind of support to deal with this increase.

So this blue line is actually the number of FTE positions that we have, and there is no reason to ask for additional ones because we can't fill them. So the green line here are the number of filled positions. So this represents the great need for automation and also methods that don't cost as much. Reagents have doubled their price in recent years twice, and so these are major issues for the hospital side of things.

(Slide.)

So just taking a bird's eye view and summarizing

sort of what we have been saying yesterday and today. Any kind of method is going to need reagents and some kind of method in which to use them. Currently the reagents comprise red cell antibodies, anti-human globulin, reagent red cells, and associated other supplies. The methods currently used, serologically used, agglutination or some variation on that as the readout.

(Slide.)

So as we have talked about over the past couple of days, some of the drawbacks is the expense and in some cases the scarcity of antibody reagents, and that the method isn't practical for performing extending phenotyping on a routine basis. So sort of the standard of care is that you type units for A, B, and D, you type patients for A, B, and D; and you match them up, and you don't worry about anything else not matching until there is something showing up as a reaction, as a consequence of not having matched before.

So in other words, we actually have been practicing a reactive type of transfusion medicine rather than a proactive type, and that is basically because of the limitations of the technology. It is impractical to try to completely phenotype serologically all units of blood and all patients and match them up using current methods.

So medically the consequence of this is first delayed hemolytic transfusion reactions, which as many of you know are not typically fatal. They can be, but that is not really the main problem medically. Probably the main consequence is just a gradual destruction of the transfused unit so the patient isn't getting the benefit of transfusion. They may require another transfusion to make up for it, and that has all the associated issues.

But I think that another medical issue really is that this whole process that we have been practicing, it creates a delay in providing blood to not just a patient who has lots of alloantibodies, but to every patient, because that one patient slows up everything else. So if a patient has because of not getting fully matched blood a patient has a positive screen, that buys another hour or so to perform an antibody identification. It might not be an hour. It could be hours. It could be more than a day. Then you need to identify antigen-negative units on the spot. They are not labeled because they have not been fully phenotyped ahead of time, so you need to pull them out.

Sometimes you can't find them in your own hospital, so you need to get your blood supplier to find them for you. That takes time. It takes time to ship them

to your hospital. Then on top of everything else then you need to perform a full cross match versus an instantaneous computer cross match that would be able to take place if the screen was negative.

I think, you know, the consequence financially of all of this is that when we look at it about 55 percent of all of the testing costs are spent working up about 15 percent of the patients, you know, that wind up having these positive antibody screens. So clearly there is a big financial savings and a medical benefit if you can better match blood to patients.

(Slide.)

So one of the reasons why that is not easily possible is what we talked about yesterday, which is the expense or shortage of many antibodies. So the first thing I want to talk about is just how you can make antibodies using -- for conventional type reactions using alternative methods such as phage display. So many of you are familiar with hybridoma technology where a mouse has been immunized with antigen. The mouse is killed. The spleen is taken out. The spleen cells are immortalized, put into culture, put into many, many plates over a period of a week or two. Each one of 10,000 wells is examined for the presence of a

clone making an antibody of interest. Those wells are identified and subcloned, and then you end up having a hybridoma cell line.

So this has been an incredible advance in diagnostic medicine. A couple of people got a Nobel prize for figuring this out, so it is hard to knock it. But in transfusion medicine there are certain limitations. Well, in any application it is labor intensive and expensive. Fairly inefficient because you are screening thousands and thousands of wells to only find a few positive clones.

You get what you get. So if you wanted an IgM antibody or an IgG antibody essentially unless you do something particularly out of the ordinary here you are going to wind up getting cells that make whatever they make, and hopefully you will get what you want.

The other thing is that the antibodies aren't human. Though we don't really care about making human antibodies as you would when you wanted to make a therapeutic antibody that you can infuse into a person, as many of you know animals like mice don't make antibodies to a lot of these clinically-significant antigens such as the Rh antigens and in some of the other clinical-significant alloantigens. The reasons why are probably just because if

you immunize a mouse with human red cells and the mouse is looking at the D protein it sees so many differences from itself that the antibodies it makes really can't differentiate D from C or E for example. It makes more generic type antibodies like an Rh17 or an Rh29 type antibody. That may be the reason. But in any case, it has really been necessary to try to immortalize human lymphocytes from patients who make antibodies to get these monoclonal antibodies, and the process for doing that have certain drawbacks which get into the comments that were made yesterday, which is why the monoclonal antibodies that we have now may not be ideal.

(Slide.)

So one way of immortalizing human lymphocytes is using the Epstein Barr virus transforming approach, which is a fairly inefficient approach. There is no good fusion partner for human lymphocytes, so trying to make hybridomas or what they call heterohybridomas by fusing human cells with mouse myeloma cell lines has a very low fusion frequency. There is a decline in antibody production and growth when you get one of these cell lines, and often they are very unstable and there is a progressive loss of human chromosomes, so it is not a very efficient process. Of

course there are many of these cell lines, and they make some of the type reagents that we have, such as the anti-Rh monoclonals are all make in one or a combination of these two methods.

(Slide.)

So what phage display actually allows you to do is do something that gets around a lot of these problems and then actually could allow you to be more selective in the kind of monoclonal you have to get around some of the issues that were mentioned yesterday in terms of the quality of monoclonals.

If you would just thinking for a second sort of a science fiction picture here, if antibodies in serum where actually physically connected to their DNA then making monoclonal antibodies would be pretty simple because if these orange antibodies in the corner -- okay. If you immunized an animal or you had a person and you took their serum and their antibodies were connected to DNA, then if these were antibodies against a specificity you wanted, you could take the serum, absorb out the specificity you wanted, either on the antigen that is in the well of a plate or against a cell like a red cell, elute the antibodies out. Though the elution would destroy the antibody, you would

have the DNA here which you could zap into some kind of cell which would see the DNA and start making more of that antibody. If this is the way life was, then if would be very easy to make monoclonal antibodies.

(Slide.)

What phage display attempts to do is recreate this in the laboratory, and the whole thing centers on this guy here named George Smith who in the mid ‘80s had this idea where if he took filamentous bacteriophage, which is an innocuous kind of phage that infects bacteria -- it is filamentous because it is long, and like any other virus particle there is nucleic acid in the middle and proteins around it. He thought, "What if I took DNA that encoded some irrelevant polypeptide and if I cloned it into the DNA or the particle just in front of the DNA that happens to encode this co-protein of the virus? What would happen?" What he found is what he predicted, is that the bacteria and the phage don’t really care if you did that, and what it does is it makes the co-protein a fusion protein of the protein encoded by this exogenous DNA with the DNA for making this protein.

So what he actually accomplished here was the linking together of the phenotype of a protein with the

genotype of a protein. So essentially if this was an antibody then he has an antibody that is physically connected to the sequence of DNA that is required to make that antibody. Sort of like having serum physically connected to its DNA.

(Slide.)

Over a period of five or six years what developed was the idea that you could actually take B cells from an animal or a person, peripheral blood lymphocytes from a human, and let's say this making anti-D or anti-Kel or whatever. You could take this material, extract the RNA, carry out a series of PCR reactions with degenerate primers, and in a couple of weeks of work create a phage display library where each particular expresses a different antibody on its surface but inside has a unique piece of DNA that encodes that particular antibody.

(Slide.)

And so if you take the library and you pan it in a well with antigen you will absorb out the penguins. I guess that is what these things look like, but you absorb out the phage that have an antibody that binds. You elute that out, and although the acid that you might elute with would destroy this antibody here, break this bond, it doesn't

matter because phage itself is resistant to this elusion. So this thing will infect bacteria and this DNA will get in the bacteria, and the bacteria will make more of these particles. This thing is called a round of panning, and you take this stuff and then do it again. After about two or three rounds essentially all of these particles you get have antibodies against the thing you panned against.

(Slide.)

So just to tell you what is actually on the tip of this, of the particle, it can either be -- if this is a regular-looking antibody it can either be just the variable region connected as one protein, the variable region of the light chain and the heavy chain; or it can actually be a Fab fragment where the bacteria actually make the FD fragment of the heavy chain and the complete light chain and assemble a --- bond in the bacteria itself, and that winds up being expressed on the phage. In either case, inside here is the DNA to encode this or the DNA that encodes this.

(Slide.)

So to summarize this process, the conventional approach for making monoclonals would be to take B cells and try to immortalize these cells in wells. The phage approach is to take the same cells, but break them open and use

molecular methods to create these libraries from which you can isolate the relevant antibody and its DNA.

(Slide.)

So some of the advantages is that it doesn't rely on immortalization. You can adapt it to make antibodies from any species, and these species all you need to know is just what PCR primers to use to amplify antibodies. It is RNA based, so you can get access to all B cell compartments. So plasma cells are rich in RNA, so their antibodies are represented in phage libraries, whereas the hybridoma technique and EBV will not immortalize a plasma cell, which is a consequence of why conventional methods for making monoclonal antibodies in blood typing haven't been as robust as one would want them to be. You can use just IgG primers if you want to get just IgG antibodies out, which have higher affinity than IgMs. The whole process is very streamlined and rapid.

It can take a month to do one of these experiments, and the antibodies themselves can be stored as bacterial stocks, as phage as particles in the refrigerator, and the phage particles themselves are capable of self-replication. So the antibodies themselves can make more of themselves. When you run out you just take a speck of them,

add it to some e. coli, and then by the next morning you have a flask of antibody.

(Slide.)

So the way we have used this --. and I have presented this before. Many of you have probably seen this. We have made antibody phage libraries, in this case from an individual who makes anti-D. It is selected on a Rh positive cell sort of as though you were doing an absorption technique in the blood bank. The process is a little more involved the way we do it. We try to do a negative absorption with Rh negative cells first, or actually at the same time using some procedure I won't get into, but a way of sort of just having the antibodies against in this case Rh just captured on these cells, having other cells that go about other things. You then elute using the same elution acid glycine you use in the blood bank. You take this stuff, neutralize the acid, and infect e. coli and grow the thing up, and that is how you can do panning on intact cells.

(Slide.)

In a typical experiment, this is from an anti-D, we got over a million anti-D clones from staring with one of these libraries that originate from 28 mils of peripheral

blood from an individual. Just looking at 83 out of over a million clones there were 53 different anti-D antibodies.

(Slide.)

How can you work with antibodies or even show that they bind to what you think? So this is your typical indirect agglutination reaction where you would incubate red cells with an antibody wash and then add a --- agent and get agglutination. So similarly what I showed you could do is take red cells and incubate it with these phage particles and then wash, and then add a commercially-available anti-phage antibody and they will agglutinate.

(Slide.)

So this is just in a microplate showing Rh negative cells, Rh positive cells with different dilutions of some of this phage; and this is a positive agglutination reaction, and this is negative. You can see the titers out in this assay quite far. I will say a little more about that in a second.

(Slide.)

But basically this is an electron micrograph of human red cell, and these are the particles I am talking about, and these have anti-RHD on the tip which is a fusion protein to that co-protein which is only located at the tip.

You can see that they are actually very large, which provides a lot of ability for secondary reagents to cross-link them, which is why it is a very, very sensitive agglutination reaction.

(Slide.)

So, for example, this is a typical gel card that many of you are familiar with, and if you take one of the gel cards there, there is just a buffer card, and you do what I did, which is to pipette in some anti-phage antibody into the gel card instead of --- reagent. Then you can show that these phage particles will work in the gel card. So this shows the sensitivity of it. If you times 10^7 that this is how many phage are added to -- this impairs and these have Rh negative cells or Rh positive cells, and how many red cells are actually added using the typical recipe with the cards, which is 1.6 times about 10^7 to the red cells. Then you can see that the sensitivity is to about 13 antibodies per red cell. So you need very few antibodies to get a positive reaction in this type of an agglutination reaction. The consequence of this is that one liter of the phage can make enough reagent to do this for to type about 500,000 units of blood, and it would only cost about a dollar or two for the bacterial media. So it is a very

inexpensive way of making reagents.

(Slide.)

From that experiment, as you can see from this and some other publications, we got many different D epitope specificities, and you can actually design panning methods so that can select for particular epitope specificities depending on how you design your pan.

(Slide.)

One feature of the system is if what you want to do, you can take the -- here is a phage particle, here is an antibody sequence for an anti-D. What you can do is you can take this sequence out and put it into a different kind of plasmid which would go into a --- cell or a 293 T cell, some kind of --- cell, which would then actually put on a complete FC domain and make it into a bivalent conventional antibody which is in this example I did, and it will function in any conventional agglutination reaction with conventional anti-human globulin. So you can to it in a tube, you can do it in a microplate, and this is with a conventional Coombs gel card just putting this material here with red cells just doing a regular typing. So this stuff you can make is indistinguishable from any other kind of monoclonal.

(Slide.)

So most of the experiments along these lines have been proof of principle studies. So to date there has been very limited number of individuals who have used this to produce red cell phage antibodies. There have been hundreds of other kinds of specificities. These are the ones that you can find in the literature. We recently got funding through an NIH grant, STTR grant, to basically isolate monoclonal --- to a whole bunch of other important alloantigens including these and some others, which will probably take place over the next couple of years. We are funded to do that.

(Slide.)

So to summarize my talk so far, so the process I have described is where you would start with peripheral blood lymphocytes. You would make a library. You would paint it on red cells, and you would get antigen-specific phage particles which you could retain as phage displayed antibodies and use in conventional agglutination assays, but use an anti-N13 type Coombs reagent. Or you can convert them to conventional IgM antibodies and use for direct agglutination or convert to IgG, and then of course if it is IgG you would use it with a conventional Coombs reagent.

So that is sort of how you could use phage display to make conventional reagents to use in conventional assays to get around some of the problems that we have with monoclonals that are currently available. But what I just want to finish off with is sort of a variant on this.

(Slide.)

Which is thee was a paper that came out a few years ago from my institution which I thought was kind of interesting. What they talked about here was taking an antibody and using a gluteraldehyde* chemical coupling a piece of DNA to the antibody. The reason that they did this and what they show in this paper is that you did ELISAs with this if you would add this to a well to detect something instead of adding a secondary enzyme conjugated antibody as in a tradition ELISA, instead you could do some kind of molecular assay that would PCR something off of this. Or in the case of what they did here was to actually use T7 RNA polymerase and make RNA off of this here and detect the nucleic acid material. The reason why they suggested doing this is because the sensitivity of this got down to being equivalent to radioactivity, as if the antibodies were labeled with a radio isotope.

(Slide.)

So what it lead me to realize is that these phage particles have DNA in them and they are physically connected, and I didn't have to do that. They came out of the bacteria that way. That is actually how they are made. They have the DNA inside of the particle that has an antibody on the tip. So it raised the question in mind as to whether you could detect the binding of one of these phage antibodies to a cell by using a nucleic acid method, and why would you want to do that. So you would imagine it would be very, very sensitive. You would require minute amounts of material. It might be more amenable to automation, plus you could think how it could allow you to actually multiplex serological typing reactions because you could have different specificities, each with a different kind of DNA tag inside the particle.

Now inside the particle are the actual sequences of the antibodies that are displayed, and those are unique. But you could actually put in any kind of tag you wanted into the DNA, the phage, anything that your DNA detection scheme could allow you to discriminate, something that might be on a microarray for example.

(Slide.)

So what I kind of called this was phenotyping by

reagent genotyping. So the idea is that you are genotyping the reagent, not the person from whom the red cells came.

(Slide.)

So to show the feasibility of this, here is a phage antibody that has D on the surface, and I put in some arbitrary piece of DNA. I call it a tag here, and here is some. By these arrows indicate PCR primers, and if you just take a particle like this and throw it into a PCR reaction, and using real-time PCR and using cybergreen dye which inter-collates into double stranded DNA as the PCR products form, you see what happens after a certain number of cycles. You begin to get lots of these tags, and then if you tell the machine at this point to lower the temperature down to 64 degrees, which is what the axis here is, temperature, and slowly raise the temperature what happens is at a characteristic temperature based on the melting point of this tag the cybergreen falls out of the PCR product because the PCR product has melted.

So the fluorescence decreases, and if you have a computer replot this data as a negative first derivative of this curve, it just allows you to see a very sharp peak here where the slop is zero basically, and that identifies a very unique melting temperature for this head. So this process

takes about 20 cycles, about 10 to 15 minutes to actually complete this process.

(Slide.)

So if you take an anti-D phage particle and you add it to by agglutination it doesn't agglutinate D negative cells. It does agglutinate D positive cells, and you do this reaction. You see that you get a very strong peak at the characteristic melting temperature of this tag. So in other words, this process can tell you whether the antibody bound to the cells or not. This process in this experiment used about 150 red cells as opposed to -- which is equivalent to $100{,}000^{th}$ of a drop of your typical drop of three-percent red cells. So of course this thing kind of lends itself to being markedly nano technology sized.

(Slide.)

So just to give you a couple of other examples before I finish. So we created three other kinds of phage that had three different tags of different lengths, and this is just showing you if you mix them together you get three peaks corresponding to each of these tags.

(Slide.)

I need to link to a file that allows me to have vertical slides so you can see it. So if we take an anti-D

with a short tag and anti-B antibody with a longer tag, and you add it to either O negative cells or positive cells, B negative cells or B positive cells, you can see what you get. Basically these cells don't agglutinate and all the others agglutinate based on either one or both of these things binding. But then if you do the process I described you either get a peak for the D reagent, you get a peak for a the B reagent, or you get two peaks to show that both of these things bound. So this shows that you can actually multiplex serological reactions using a DNA-based readout.

(Slide.)

But the other possibility is, you know, if this whole thing can take place with only 150 red cells it may not really be necessary in one reaction to do 20 different phenotype reactions. Perhaps you can have in a very tiny kind of a chip that has these liquids in it each having 150 red cells you could use this multiplexing type ability to instead only type for one antigen, but have internal negative and positive controls.

(Slide.)

So for example, we have phage particle here with a short tag with an anti-RH17 antibody on it, and this is something came out of some studies that Marion Reid and I

did a number of years ago from a macaque that was immunized with human red cells, and this was isolated using phage display. If you take a particle here that has a slightly longer tag and this is an antibody against something in the skin, so this is totally negative control for red cells, then here is our anti-D with a longer tag. You can see that this reagent will agglutinate D positive and D negative, but not Rh null. This one will agglutinate anything, and this will only agglutinate D positive cells. Individually you can see that they will each give a curve in a different place.

(Slide.)

So if you mix them all three together and incubate it with D negative or D positive cells, you get agglutination in both cases because of at least because of this positive control. But when you look at here the positive controls, positive in both and negative control is negative in both cases, and then only Rh positive cells have a peak in that position. So this allow you in every single individual serological reaction to have a negative and a positive control to tell you that things are working.

(Slide.)

Just for fun, what I did was to take a phage

particle and put staph protein A on the tip, and staph protein A is something that belongs to the FC domain of IgG. The question is could you kind of use this method for doing screens or panels, so this is what this particle would look like. It would give you a peak here based on this tag.

(Slide.)

So here are six reagent red cells that are mixed with a patient serum that contains anti-D, and the one, two and the three cells are D positive. These are D negative, and so I have them drawn here with the antibodies on the appropriate cell. If you run them in a gel card this is the typical agglutination reaction you get, and if you instead add a staph A displayed, protein staph A displayed phage particle and used the molecular readout, I am not sure how clear you can see this, but there is a peak here, a peak here, and a peak here and these are negative. So in this case you can use these things for indirect agglutination reactions.

(Slide.)

Here is the same thing with anti-E containing serum, and this is the gel card result and the result you get with this assay.

(Slide.)

So, you know, this is your standard tube, your gel card, and what we are in the process of doing is designing these lab-on-a-chip type devices that have multi-channels in it where you could have many reactions occurring in parallel. They have these --- gates and so forth within the chip that can be used for when you need to wash the cells and so forth to removed unreactive reagent. The PCR reactions, whatever you want to do molecularly, can take place in these chips.

(Slide.)

So, you know, one possibility could be that you could multiplex typing reactions and get a profile of what antibodies bound based on the DNA inside the particle. Or, as I said before, each channel would just tell you about one specificity, but would have internal/external controls.

(Slide.)

So to summarize, what I showed a few slides ago was this idea of using phage display to create either phage antibodies that you could use in agglutination assays, or phage antibodies that you convert to regular antibodies. One thing I didn't mention is that if you -- you can take monoclonal cell lines that exist right now. Instead of having to start here, you can actually just take the cell

line and extract the -- make RNA and PCR at the antibody that is made by them, and then put them into the system if that is what you wanted to do. There wouldn't be much of a reason to do this unless you wanted to convert an IgG into an IgM or something like that.

But what I want to propose here is that the anti-phage antibodies could be used not only in convention agglutination reactions, but in this reagent genotyping approach. So when we talk about phenotype versus genotyping, one question is what would you do phenotyping or serological reactions for. But separate from that, is there some way of combining the technologies together in terms of the device, the machine, the way things are read out. So really what genotyping is doing is it is starting with DNA, genomic DNA, but essentially what it is doing is creating an array of different pieces of DNA and then asking which ones are there. Which is the same thing as having a bunch of phage display antibodies that had been bound by a cell and eluded off and asking what tags are there that came from those reagents.

So the endpoint really can be the same, whether it be -- regardless of what kind of molecular technique you might be using for seeing what DNAs are there, whether it is

a microarray or some other kind of method. So this may be a way technically of at least having the technologies and the readout combine together at the end, even though the input in one case would be genomic DNA and the input in the other case would involve some serological -- some cell antibody reaction initially.

(Slide.)

I would just like to acknowledge some of the funding sources that have lead to this work, from the National Blood Foundation to NIH, to some Pennsylvania-based biotechnology support funds. That is my talk, and it is time for lunch. Thanks.

(Applause.)

MS. KOCHMAN: I had us scheduled to come back -- to leave at 11:30 and come back by 12:30. I don't want to take a half-hour away from you, so how about if we come back in 45 minutes?

(A luncheon break was taken at 12:07 p.m.)

A F T E R N O O N S E S S I O N

(12:55 p.m.)

Current FDA Processes for Bringing Products to Market

Sheryl A. Kochman

MS. KOCHMAN: I would like to get started again. Before I do, I would like to remind anyone that if you need transportation to one of the airports please get with Rhonda at the registration desk, and I have also been asked to remind you again to fill out your evaluation form. We have also got a pair of sunglasses that were found in a chair in the lobby, so if you know anybody, if they are yours or if you know whose they might be, they are up here.

My first talk is probably going to be primarily of interest to anyone involved or associated with the manufacture of an in vitro diagnostic product, but some people, some of the users might find it useful to understand what FDA does and does not do in terms of premarket review of products.

(Slide.)

So the first question and the thing that I find a lot of people don't know is that IVD reagents and instruments are medical devices, at least in the United States. They are classified to be medical devices.

(Slide.)

We get that from the FD&C Act, and really all you need to look at on this big slide full of words is instrument and in vitro reagent. But pretty much you will notice that we have thrown everything we can think of into this definition of a device, so if it is a thing it might be a device.

(Slide.)

If it is a think that is also one of three other defining topics, and it is a device, and the reagents and instruments fall in the second class because they are intended for use in diagnosis of disease or other conditions or in cure, mitigation, treatment or prevention of disease in man or other animals. Basically we are trying to prevent hemolytic transfusion reactions, so that is why these are devices.

(Slide.)

There is a whole other part, and basically in

plain English this whole paragraph means it is not a drug.

(Slide.)

The other thing is that a lot of people may not realize that IVD reagents can also be biological products.

(Slide.)

Again, here is a whole big, huge paragraph, the last part of which says they may also be biological products subject to section 351 of the PHS Act. This is out of 809.3(A), which is part of the medical device regulations.

(Slide.)

So you might ask when is a device also a biologic. Biologics regs define a biologic product to mean any virus, therapeutic serum, toxin, antitoxin, or analogous product applicable to the prevention, treatment, or cure of diseases or injuries of man. Again we have thrown a lot of things into that definition.

(Slide.)

The next question might be, well, how does FDA regulate medical devices. Interestingly the Public Health Service Act was the first act that came along to regulate things. That was in 1912, and the net act that came along was the Federal Food, Drug, and Cosmetic Act, or the FD&C Act of 1938. Interestingly you will notice that the medical

device amendments to the FD&C Act did not come along until May 28th of 1976. That is important because it is part of the reason some devices are also biologicals. Prior to 1976 the government didn't have a definition for a device and they weren't regulating devices. So those products that were available on the market prior to 1976 were either unregulated or they were regulated as a food, drug or cosmetic -- or a biologic. Because of the PHS ACT and because blood grouping reagents and the like are used in testing blood, they were being regulated under the Public Health Service Act.

When the medical device amendments came along, we realized that it fit that definition as well, and so the other medical device acts and amendments have been applied to licensed biologicals that, but IVD since then. There is the Safe Medical Devices Act of 1990, the FDA Modernization Act of 1997, and the Medical Device User Fee and Modernization Act. We affectionately call it MDUFMA. We have to be able to pronounce everything.

(Laughter.)

Of 1992, and that is the act that allows FDA to assess user fees for the premarket review of medical device submissions. The regulations that cover medical devices

include 21 CFR part 600 if the IVD is licensed, and 21 part 800 if it is licensed or not.

(Slide.)

The way in which a device is regulated is based on a classification scheme. This classification scheme is to some extent a risk-based scheme. A class one device is a device meeting the lowest level of regulation. It has been determined that general controls are sufficient to provide reasonable assurance of safety and effectiveness for their intended use, and the device is not life supporting or life sustaining, or for a use which is of substantial importance in preventing impairment of human health and which does not present a potential unreasonable risk of illness or injury.

(Slide.)

I mentioned that the second bullet says that general controls are sufficient. General controls are defined in the regulations. They consist of registration and listing by the device manufacturer, adhering to good manufacturing practices. It may include premarket notification or a 510(K) submission. There is a prohibition of alteration, misbranding, or manufacturing banned devices. There is a requirement for record keeping and a requirement for reporting of device failures.

(Slide.)

A class two device is a device that is subject to special controls in addition to the general control requirements in order to be able to provide reasonable assurance of safety and effectiveness for their intended use.

(Slide.)

Special controls that are in addition to those general controls are performance standards. It was the intent when the medical device amendments went into effect that there would be performance standard promulgated for devices as they were classified. This is one of the things that until recently has not really been accomplished. I will touch on that a little bit more later. It also can include post-market surveillance and/or patient registries and/or guidelines and guidances from FDA. It definitely includes design controls and may include tracking requirements.

(Slide.)

A class three device is something for which we believe there is insufficient information to determine that general controls and special controls together are sufficient to provide reasonable assurance of safety and

effectiveness, or the device is life supporting or life sustaining, or for use which is of substantial importance in preventing impairment of human health, or the device itself presents a potential unreasonable risk of illness or injury. The risk ran away there.

(Slide.)

In plain English, a class three device is one that has no established predicate. I will explain what a predicate is in a few minutes. The device is associated with some sort of high risk, or because it is a new device and we know little about it, it raises new types of issues of safety and effectiveness.

(Slide.)

I tried to put those three lists together on one chart, and generally what you see is the list of things that apply growing. They are a little bit color coded so that you can see that the top items apply to all of them. Some of these additional standards apply to class three. But the main thing that is important on a class three device is that we require valid scientific evidence, well-controlled studies, and we do allow some use of documented case histories in support of these products.

(Slide.)

Now that you know the classification, you need to know the pathways to market. Not all devices are reviewed by FDA any longer. As part of FDAMA* the Congress asked us to look at our devices and see if there were any that we could potentially stop looking at prior to allowing them to go to market, and so most class one devices are currently exempt from the requirement to submit a 510(K) and some class two devices are exempt from that requirement. So as long as the manufacturer believes that their device is similar to something that is already on the market and they adhere to all of the general controls, they can proceed to market without FDA review.

As I mentioned, a premarket notification is usually referred to as 510(K). It comes from section 510(K) of the FD&C Act. There is also premarket approval or a PMA, and these are for those significant risk devices, the higher-risk devices, and of those higher-risk devices those that are considered significant risk devices require an investigational device exemption before they can be shipped even for use in studies.

Another path to market is the product development protocol. This is sort of a PMA, just submitted in a different format. There is also humanitarian device

exemptions. These are like orphan drug submissions. We now have a category of product called analyte specific reagents that you can come to market through that, and then we have the licensure of BLA. With the BLA the submission of BLAs requires an investigational new drug application prior to submission of the BLA. Non-exempt IVDs are those that require it. There are some that are exempt. You might ask why an investigational new drug submission. It is because we were asking for these before we had medical devices. It is analogous to an IDE in many ways.

(Slide.)

The 510(K) process, as I said, comes from section 510(K) of the act. This process requires that the manufacturer demonstrate substantial equivalence or that they are substantially equivalent to another device on the market. The device has the same intended use, similar technological characteristics, and no new issues of safety and effectiveness as compared to something else that is legally on the market. There is a 90-day review clock, and from FDA's point of view there are lots of limitations in that review. It is basically a paper review. We don't have any inspection authority. We don't have any ability to perform hands-on testing. There currently are no

performance standards. Most of the products in this area don't even have a gold standard, and there is a lot of bias in the process.

(Slide.)

The major elements of a submission, the exact criteria for what has be included in a 510(K) submission are at 21 CFR 807.87. But the main thing that the reviewer is looking at is the intended use and indications for use statements, the performance characteristics of the device; and they are comparing those things to the labeling, primarily the package insert.

(Slide.)

Substantial equivalence is similarity of a new device to one that is or was already legally on the market, which we call the predicate device. Note that I say was. It is possible for a manufacturer to come in with a device that was legally on the market but for whatever reason the first manufacturer has decided it was not really a feasible device to stay on the market. It wasn't profitable for them or for some reason it came off the market voluntarily. A new manufacturer is allowed to use that device as a predicate.

One thing that everyone needs to realize is what

substantial equivalence is not. It is not a determination that the new device is exactly the same as the one that is or was already legally on the market, let alone that it is any better than the one that is or was on the market, and it is not an FDA approval. It is simply a FDA review that says this appears to be similar to something that is out there.

(Slide.)

The PMA process we get from Section 515 of the Act. In this case approval is based on reasonable assurance of safety and effectiveness based on valid scientific evidence, and it does say reasonable assurance. It doesn't say 100 percent assurance. There is a 180-day review clock. We have the same limitations in review -- or similar. Lack of performance standards, lack or gold standards. In this case there is a lack of historical information because there is no predicate, and again we have lack of the ability to test it ourselves.

(Slide.)

What is required for a PMA is described in 21 CDR 814.20. Again we get the intended use and indications for use statements, the performance characteristics and the labeling. But in this case we also will get clinical and/or field trial data, and we also have the opportunity to

perform a pre-approval of the manufacturing facility.

(Slide.)

The BLA process comes from the PHS Act. We are looking for safety, purity and potency in this case. A standard application, we are allowed 10 months to review that. A priority application is reviewed in six months, and a priority application is something that both manufacturer and FDA agree is in the best interest of public health to get to the market on a more expedited path. There are supplements to BLAs, and for devices we have between -- some of them are four months, some of them are six months, and some of them are 10 months. In terms of limitations in review for the BLA process we pretty much have very few limitations on our review process, and if there are any it is primarily because they are new, innovative products that we don't know enough about.

(Slide.)

All of the elements required in the submission are described in 21 CFR 601.2. The major elements again are the intended use or indications for use, performance characteristics, labeling, clinical or field trials. In this case we get conformance lots. We get actually have the product in our hands and do the testing, and we have the

opportunity to do a pre-license or pre-approval inspection of the manufacturing facility.

(Slide.)

I tried to condense all those into one slide, and so the bar. I couldn't think of another word to call it. The bar for 510(K) is substantial equivalence; PMA is reasonable assurance of safety and effectiveness; and BLA is safety, purity, potency, and in some cases also specificity and ---.

I am not going to go over all of this in the interest of time. One of the other key areas, though, that is different is in post-market. Products that go through the 510(K) process are generally only inspected for cause post-marketing. For cause means that there are reports of problems with the device, and the field goes in to follow up to see if the manufacturer has handled those problems appropriately or if there are other problems, if that is just the tip of the iceberg and there are other problems that need to be addressed. But basically the are not on any scheduled basis.

Under a PMA there are periodic inspections. The periodicity is based on the risk associated with the device. It varies, and there is also a requirement for annual

reports of changes in the process to come to FDA.

For a BLA we have biennial inspections. We have annual reports just as with the PMA, but we have continued lot release, which means that once we get the conformance lots every lot that a manufacturer makes they must submit it to us. We must okay it before they can distribute it, and we have a requirement for supplements. Which are applications or submissions to FDA for certain kinds of changes, and the manufacturer has to wait for our approval before they can implement those changes.

(Slide.)

Current immunohematology products are either 510(K) regulated or BLA regulated. We don't currently have anything regulated under the PMA process, and if you look you will see that the 510(K) products are HLA kits, a lot of those accessory kinds of reagents that you are using in a reference laboratory, and some instruments. Automated blood grouping and typing instruments are a 510(K) class two. Centrifuges and cell washers fall under the 510(K), but the reagents, that manufacturer is allowed to put a license number on or the blood grouping reagent red blood cells and anti-human globulin.

(Slide.)

And a word for any manufacturer, we really manufacturers to meet with us before you even start your clinical or field trials. We want to make sure your test plan covers all areas we would want to see covered in a submission. We want to help make sure your pre-market submission is complete. One of the complaints we get is FDA doesn't move fast enough on anything we submit. The speed at which we can review something is proportional to the amount of work you put into it or don't put into it. So the better your pre-market submission is coming into us the quicker we are likely to be able to get through it. We also want to make sure that you don't do anything that we wouldn't necessarily think you need to do.

In order to request a meeting with us I would refer you to a REG SOPP 8101.1 scheduling and conduct of regulatory review meetings with sponsors and applicants. Our website is there, and then after you have looked at the website and see we spell out exactly what you need to have ready before you can even ask us for a meeting. So once you have that information then you can contact me. I will likely assign it to one of my staff to set up the meeting, and that is that end of that one. I will move quickly to my next presentation.

Review of Current FDA Guidance

by Sheryl A. Kochman

MS. KOCHMAN: The next presentation, review of current FDA guidance. I am not actually going to review guidance. I know that one of the difficulties many people have is finding the information that FDA makes available to the public. The different websites have different ease of use, and I hope to provide you with a little bit of help finding things that might be of interest to you, but of course I have several disclaimers here.

(Slide.)

The information provided on the following pages is not intended to represent an all-inclusive list of guidance documents pertinent to the manufacture and use of molecular methods in immunohematology. I have included some guidances only to provide information regarding FDA's current considerations in regards to the areas mentioned. Especially I have included some documents that clearly state that they are draft which basically means they are not things that we can rigidly suggest that you follow. It is to let you know which way we are thinking on things, and I may have unintentionally omitted some things that you may find would be helpful. I hope not, but that obviously

exists.

(Slide.)

To start off, there are several homepages that I would recommend people be aware of, and each one of you will see something that may be of different use for you depending on the situation you are in, whether you are a manufacturer, whether you are a user, whether you are trying to help a manufacturer by doing field trial testing for them.

So I have got the CBER website; another wealth of information is available from CDRH's office of In Vitro Diagnostic Device Evaluation and Safety, OIVD for short. They handle all of the in vitro diagnostic reagents that are not related to blood testing. So if there is any clinical implication for an IVD it goes to these people. Their website has links to all of the IVD guidances that are available. They also have links to -- it says IVD standards.

I want to clarify that also part of FDAMA was the requirement that FDA determine what national and international standards are available to manufacturers of devices and determine if any of those provide information and guidance that is acceptable to the FDA in terms of developing and manufacturing a device. So anyone can

nominate a consensus standard for recognition by FDA. The process goes through CDRH since they are the primary center for handling devices. They involve us on an as-needed basis. But they look at the standard that has been nominated. They determine whether or not that standard results in testing and documentation that a manufacturer can use to prove that their device is safe and effective or reasonably assures that it is safe and effective, and they can recognize it in whole or in part, or they can determine that they don't recognize it.

The link to IVD standards does not actually link to the standard itself. It links to a list of standards that FDA has accepted or has recognized, and that way a manufacturer when they make their submission to FDA can state, "I am conforming to the NCCLS guidance on," something or another. If that guidance is one of the ones we have recognized then we have a little bit more of a warm and fuzzy feeling about what that manufacturer is doing.

Another really helpful website for manufacturers' devices is CFRH's device advice website. I have you the very, very basics on device classification and on pre-market submissions. This website covers everything except the BLA process. The BLA process is unique to CBER and so you would

have to come to us for that. We do have, as I mentioned, medical device user fees now, and so anyone considering bringing a device to market really needs to go and see what there is about the medical device user fees.

(Slide.)

We have a few guidances that are specific for immunohematology reagents. There is no web link here because they are so old they are not on the web, and you will also note that they are all also still draft. I am embarrassed to have to have say that. But we have the recommended methods for blood grouping reagents evaluation. That basically is the document that we encourage manufacturers to consult if they are manufacturing typical blood grouping reagents, and it is the methods that we advise them to use when they are doing their lot release testing. We have a similar document for anti-human globulin reagents.

We also have a document that was developed in conjunction with a 1990 workshop that is called "Points to consider in the design and implementation of field trials for blood grouping reagents and anti-human globulin." I make reference to it because that guidance talks about things like the number of sites we want to see you include

in your testing, the kinds of sample conditions you need to consider including, and all sorts of things like that. While these documents are not available on the website, they are available from CBER's Office of Communications, Training and Manufacturing Assistance or from me or my staff.

(Slide.)

One that is on the web is guidance for industry content and format of chemistry, manufacturing, and controls information and establishment description information for a biological in vitro diagnostic product from March of '99. We used to have one establishment license application and then a separate product license application for lots of different kinds of biological products, and in an effort to simplify things we did away with the establishment licensing application and include that information in the biologics license application. Then we have only one form for getting a biologics license application, but to help the manufacturers in completing the form and then building the dossier or the submission we have these guidance for industry on what kinds of information goes in that part of the submission.

(Slide.)

There are a number of documents available on

molecular tests, none of which are really specific to this area. I am pointing them out because for one thing I want to show that FDA does recognize that molecular testing are coming or are here, and they may provide some little tidbit of information that could be helpful to you. So there is this one from 2005. I am not going to read all these because I am trying to get everybody caught up here.

(Slide.)

Now this one is interesting because the draft guidance for industry and FDA staff, pharmacogenetic tests and genetic tests for heritable markers, I included this because I thought it might come up. Interestingly Marion indicated that the state of New York has decided that their blood group genotyping is not a test for a heritable marker, so I think that that is interesting. But it is there for you to read if you want information.

(Slide.)

Another area that is really coming on the scene is the whole concept of personalized medicine and matching the drug to the patient, and some of these other guidances are directed at that. I think you will see that in the address you can tell which center issued it. A number of these are from CBOH.

(Slide.)

Here is one on gene mutation detection systems, factor Lieden DNA mutation detection systems. I thought that some of the information about what FDA is looking at in terms of mutations may be helpful to some people.

(Slide.)

In some ways these are a little more dated guidances, but we do still have on the books some guidances for biotechnology products. These ones are out of CBER.

(Slide.)

There is a general IVD guidance for industry and FDA staff, but I actually think that users will find this helpful also. It is for use of symbols on labels and in labeling of in vitro diagnostic devices intended for professional use. This was issued in 2004, and while it is CDRH listed guidance, CBER was also substantially involved in this document.

Because the European Union's in vitro diagnosis device directive states that if a manufacturer places labeling statements on their label in a give language any member state may require that that labeling statement appear in the language of that member state we -- industry came to FDA and said we are going to have problems with this. We

have limited amounts of space on labels and in labeling, and if we have to put labeling information in 14 different languages we are not going to have enough room for people to be able to read the information. So because the current requirements are that there is a specific requirement that states that if a statement is to be included on the labeling it must be included in English there was concern about whether or not we could have kinds of concessions about that.

Because one of the things that the EU recommended was that we incorporate universally acknowledged symbols in the labeling rather than having to have 14 different languages. So this guidance recognizes certain symbols as being universal. They have been tested in an American market to see if they are indeed recognizable, and they may be helpful to users as well as manufacturers.

(Slide.)

There is interesting guidance available on how to use the data that you have gained from your studies. Statistical guidance on reporting results from studies evaluating diagnostics tests. It is still listed as a draft guidance for industry and FDA, but is relatively currently, from 2003, and I think it provides a lot of explanation of

why things should be worded certain ways.

(Slide.)

There have been a number of things mentioned here on informed consent. The most recent document that has come out on informed consent, it pertains to the use leftover specimens. For example, when you are in a blood establishment and you have processed all the blood that you have collected, you have pilot tubes left over. Can you use those samples in support of some testing, and if so how do you do that? So this guidance is particularly relevant to anybody who is considering doing testing in support of a manufacturer's submission. Again, it is on CDRH website, but we had input into it as well.

(Slide.)

These other guidances on informed consent are actually on the webpage of the Office of the Commissioner, so they are at a much higher level, but I would suggest that you take a look at these. The Declaration of Helsinki is used in terms of I have had some people ask questions about whether or not we will accept foreign data. The Declaration of Helsinki deals a little bit with that.

(Slide.)

Here are some other documents that are important

for you to have.

(Slide.)

Then there are also some webpages that were available that if you go to these webpages there is are a whole bunch of other links to things that I couldn't even anticipate whether or not you would be interested; but information is power, so now you have the information.

(Slide.)

We have got a number of guidances on clinical or field trials. Guidance for industry on acceptance of foreign clinical studies. Another equally important guidance for financial disclosure by clinical investigators. Guidance for industry on computerized systems used in clinical trials. I think these three are all pretty important for anyone considering doing studies to support a manufacturer.

(Slide.)

There is one current document that I wanted to include because I anticipate that as any of these technologies come to market there is going to be instrumentation that goes with it. This is included simply because it is one of the most current guidances on instrumentation. I don't know yet how useful it will be for

these products.

(Slide.)

The other thing that is clear is that these products will probably have software associated with them, and we are following this most current guidance for industry and FDA staff on the content of premarket submissions for software contained in medical devices. So this one is equally important.

(Slide.)

This one is of concern. It is actually not a guidance document. It is a compliance policy guide. This is what is made available to FDA investigators when they go out to perform inspections. It is on commercialization of in vitro diagnostic devices labeled for research use only or for investigational use only. Again, this is listed as a draft, but the essence of the guidance is that a path for an IVD to market should have three somewhat distinct phases.

There is the initial phase where you have an in vitro diagnostic and you are trying to determine if it has any potential use, and so you are doing research and you are collecting preliminary data. That is the phase where it is for research use only, and after you have determined that your device -- you believe your device is going to

especially useful for a particular intended use.

Then you move to an investigational use study where the intent of the study is to determine "Have I got my intended use current and what are my specific performance characteristics? How well will my device do what I want to say it does?" So this is where you get into sensitivity, specificity, and that sort of thing.

Then the next phase is that once you have done those studies we expect that you are going to bring that product to FDA for premarket review because you want it to be an in vitro diagnostic device that can be used in clinical studies. This guidance gets into some of the explanations of what kinds of labeling are required at the various stages, what kind of labeling on the device is required, and what FDA's expectations are. I am just going to leave that point at that.

(Slide.)

Thee are some very new draft guidance available on home brew. I think that it probably is a good idea for people to take a look at these, and since they are draft and since they are so new you have the opportunity to provide your input on these. They do I think answer a lot of ambiguities about what exactly are ASRs, when is something

an ASR, when is it an IVD and that sort of thing. So I strongly encourage you to look at the ASR guidance.

The second one I would not have had any idea what this guidance was about just by reading the title of it, but this is software that gathers information, analyzes it, and then gives a diagnosis or a result. So this is more related to software products.

(Slide.)

And probably some of you may know about these already, but if you want automatic alerts for new postings of information you can go to the CBER mailing list subscription or to the CDRH mailing list subscription, and you can get daily updates about what has just posted or you can tailor it. The CDRH one you can tailor to get it weekly instead of daily or monthly, and that is a useful thing. The availability of the transcript from this meeting will be announced that way. We can though say that it will available within a couple of weeks, but if you want to know the exact date that it comes out you can subscribe to this list and you will get an email about it, and that is all I have for that.

(Applause.)

Where Do We Go From Here?

MS. KOCHMAN: I think everybody is anxious to have more discussion of where we are going with these things. If I could have today's speakers all come down if you are still here, and there are some people who have had to leave.

DR. WHITSETT: Sheryl, Carolyn Whitsett over here in the corner. There was a question that I wanted to ask, and it relates to testing of blood donors for hemoglobin S and what we should do about those. At least I believe, Sandra, in your discussion you mentioned that perhaps we should be informing donors that they were S positive. The first thing I would like to do is say that I work with some colleagues who take care of patients with sickle cell anemia, and the feeling in the sickle cell community is that having sickle cell trait is not something that identifies the disease. So they worked very hard to have people who have sickle trait accepted and understood that that is just a normal variant and they are not symptomatic. So given that that is the way many communities visualize having the sickle cell trait, what are we currently doing at the Red Cross in terms of notifying donors if they turn out to be hemoglobin S positive although they are normal individuals otherwise they wouldn't be donating, and how do you see

having molecular testing changing what you are doing?

DR. NANCE: Okay. First of, the Red Cross does not have an organized approach to this, and it varies in what community you are in, so I can't speak to what your community does. Second, I don't think the change that I see in just the reading about genetic testing versus genetic screening, we haven't changed the genetic. We are already doing screening on our donors, and that doesn't seem to fall into the notification realm. However, with the molecular testing it looks like we will be doing diagnostic testing for hemoglobin S as opposed to just screening for sickle hemoglobin., so I am not sure how that flies. I mean, our community actually had a meeting both in Philadelphia and Washington, DC, and the sickle cell groups that were in attendance did want to know and did want to be notified. So the idea would be that you would have to for them provide some sort of a counseling or a recommendation to go to a place, an d we did have an 800 number to refer to the Sickle Cell Disease Association of America, which has a comprehensive approach to community sort of notification and counseling. So the answer to your question is I don't know, because we are not changing. We are doing hemoglobin S screening for sickling hemoglobin right now. We are going

to change if we do molecular methods to doing hemoglobin S I guess diagnosis or testing that will tell people if they have AS or SS. Obviously probably not SS.

DR. WHITSETT: Well, I guess that was my point, that they are not going to have SS, otherwise they wouldn't be donating.

DR. NANCE: We would think not.

DR. WHITSETT: They wouldn't meet the hemoglobin requirements. There are no people walking around with SS who would have a hemoglobin that would get them past the finger stick or whatever they are using. There may be some patients with SE --

DR. NANCE: I think there are. Well, yes. Yes.

MS. KOCHMAN: My one comment would be that in general it is FDA's expectation that if you obtain clinically relevant information from any of the testing you are doing it is incumbent on you to pass that information along. So I guess it is, you know, your question of is it clinically relevant.

DR. BELLISSIMO: I guess I would argue I know Marion mentioned how the DNA -- the polymorphisms in red blood cell antigens don't count as genetic disease markers, but I think clearly in this case you are crossing the line

in that when you include that test you are doing a genetic disease test and that that would require you to consent to that person you are going to do that. I don't think you should test them for a genetic sorter without their consent, nor considering what counseling ramifications such a test may have.

DR. NANCE: And I forgot to mention that in many of the articles that I read through hemoglobin S testing was referenced as one of the considerations, but it was really more to the third category, which was reproductive diseases or reproductive -- yeah, decisions, that sort of a thing more than potentially the diagnostic implication, because diagnostic was the most severe one.

DR. WHITSETT: Well, from these comments it sounds to me like we are the very least before the screening is implemented on donors using a genetic-based testing that we will need to have some discussion with the sickle cell community as well as the FDA, because patients with sickle cell trait, individuals with sickle cell trait don't consider themselves to have an abnormality, and sickle cell trait has not -- with the exception of maybe a form of hematoria* and what happens when you go at very high altitudes, been associated with clinical problems.

So I have some concern about what the impact of this kind of testing may do with our recruitment of minority donors and how this will be perceived. So perhaps some community feedback from the sickle cell community as well as the FDA's expectations need to be brought to the table to formulate how blood centers would proceed in doing this. Because the information that you are getting is exactly the information you get now from using a test that looks at, you know -- chemically at the presence or absence of S hemoglobin. So you know exactly the same thing, but you have looked for it in a different way, and it sounds like we need to change what we are going to say to donors because we have looked for it in a different way. Because I would guess that blood centers are not notifying normal blood donors that they have sickle cell trait, or is that not correct? It is highly variable. There is no consistent policy.

DR. NANCE: It is variable and it is highly, but I think we are looking at a more precise test now. Instead of just sickling hemoglobin, which primarily will probably be hemoglobin S, and it is listed. The kit manufacturer lists it as a screen assay which needs to be followed up by electrophoresis or other studies. This wouldn't be. This

would seem to be a little more precise to me, but you are right. The interpretation is the same. The label on the blood is the same, and in that way it is the same, but I think it might be different in the patient than for the donor.

DR. STRAUSS: Donna Strauss, New York Blood Center. Just for people's knowledge, New York Blood Center is not notifying donors when they test for sickle positive trait. In fact, we considered it and we spoke to some clinicians who felt that it wasn't necessary, that the people in the African American population probably knew their trait and we were truly just testing for labeling of certain products for the safety of the recipients. So we are not notifying them.

DR. MOULDS: JoAnn Moulds, Shreveport. I would like to back up the statement for a new blood center. We following NIH guidelines test all units transfused to sickle cell patients for hemoglobin S by sickle deck screening, and we do not report those results to the donor, nor do we get specific informed consent for that.

DR. SIEGEL: I have a question. So you are talking about things that are done to donors that might be diagnosing a disease, right? That is what we were just

talking about, the sickle cell.

MS. : The carrier state.

DR. SIEGEL: Oh, the carrier state. Does that carry over into finding things out in the course of donors that might put them at risk for something? For example, suppose the donor center discovers a very high anti-D titer. So that is not communicated to the donor, but it would be of interest to the donor if it was a female thinking of getting pregnant.

DR. WHITSETT: It is highly likely that most blood donors walking around with a high anti-D titer don't know about it. It is unlikely.

DR. SIEGEL: I don't know about that. Or anti-Kel.

DR. NANCE: And I have another example in the talk, was anti-HBA 1A negative with an anti-HBA 1A. I mean, that is clearly, you know, something that might.

DR. WHITSETT: Well, people don't routinely screen for anti-platelet antibodies unless there is an affected neonate, but obstetricians sort or routinely if a woman is pregnant or anticipating pregnancy will have done a blood type and antibody screen. That is pretty straightforward.

DR. NANCE: I would think that a lot of blood

centers are screening for HBA 1A negativity and then following up the negative females with an antibody screen to make sure they don't have the antibody as well. I saw Sue Johnson shaking her head yes, so I think that is really common across the country is to screen the phoresis donors for HBA 1A.

DR. WHITSETT: So she was shaking her head yes, they do it all the time?

MS. : It is not routine, but if we do run across we would, sure.

DR. WHITSETT: So you inform the donors if you --

MS. : Right. Yeah.

DR. WESTHOFF: I would assume the test could be turned off and not used or at least the results ignored or not interpreted since we are talking about specific test that happens to be on one manufacturer's chip.

DR. YAZDANBAKHSH: Yes. On the beadchip assay the format right now if you don't want to look at such that could be turned off. Any antigen including HBA.

DR. NANCE: Then there is the HBA 1A question as well then, isn't there?

DR. WESTHOFF: I have a question for ---. Are --- able to be processed by your system?

DR. YAZDANBAKHSH: Yes. Well, no. Sorry. Let me back up. I was thinking about poly-transfused patient samples. --- samples we did try to extract DNA, and you were able to extract some DNA, but routinely all our analysis, that was done with donor patient sample. They are not --- samples. They are not --- sample, so these are whole blood samples.

DR. MOULDS: I could address that also because we specifically looked at that, quite unknowingly when I first went to Shreveport, but I tried very hard to extract DNA from segments and couldn't get a darn thing and found out we were reducing everything. So in our experience, no, you can't get DNA out of leuko* reduced units. We go to the pilot tubes.

DR. WESTHOFF: My question was specifically because I have been told it is more sensitive, so we certainly know we can't get enough also out of it per the manual methods, but had anticipated maybe the BioArray or the chip methods would be more sensitive.

DR. YAZDANBAKHSH: Well, in certain cases where they are not really from some of our collaborators where we received the blood they are leuko depleted or maybe they are leuko reduced in some other way. You will be able to

extract some DNA, but on a regular basis no.

DR. FIGUEROA: Delores Figuero from --- Systems. We looked at that, too, and I don't remember exactly our results, but I believe the amount that was detected was really minute also.

MS. : This question is for Dr. Bellissimo. Do you know if the CAP plans to have anymore samples for blood group genotyping available? We are one of those nine to 10 people who subscribe because -- and at the high cost and have petitioned yearly the CAP to at least break the D typing away from the rest. Can you give us a little background of why it was incorporated with genetic testing to begin with and what is the possibility of it moving out in the near future?

DR. BELLISSIMO: I think the reason it is with the genetic testing is just totally historical way back when they started this. It was a marker that people were doing by genetics and probably mainly concerned there that it was being used for prenatal, at least in the type of genetics laboratories. So that is why it is in that survey, and I know we had comments back and I assume a lot of the comments I am hearing about are the ones you made when you called in to CAP.

So, you know, part of what I tried to find out a little bit with Sue Johnson's help is try to -- you know, the first question they kind of ask is if we did break out such a survey, you know, how many people would participate in it. Because unfortunately that nice process I described I think in proficiency testing with all the quality control materials and administrative support and all that kind of stuff is a costly process, and so there really has to be certain number of people who would participate to make it worthwhile in doing, so maybe. My guess is from what I have seen is that number may be around 20, but I don't know if you would have a better estimate. Certainly that would help matters.

Then the other question I think now given if some of this other stuff goes forward, would there be a greater need or greater participation because of this other chip work and things. That would require an effort here, and I think, you know, if we had that kind of information we could move forward. Certainly being on that committee I am in a position to help out on that part, and I think now it is a matter of, you know, what kind of numbers would be involved.

Of course, we would have to build the whole quality control stuff in. But, you know, as I said there is

a lot of infrastructure there to that. I mean, there are tons of cell lines available and a lot of them by race, so some of it may be just plowing through them like African American samples and things like that to see how many of these we can identify, but they are completely uncharacterized for those kind of gene markers, though there are lots of different population-based controls there.

DR. FLEGEL: Is there an option to internationalize this approach? Because there are many laboratories worldwide to do that if you separate and fractionate it into several different approaches. Then there might not be enough with each proficiency scheme. Flegel from ---.

DR. BELLISSIMO: No, I don't think there is any reason why it couldn't be, and I now a lot of people who do participate in our genetics survey are not from the US. A lot from Europe and other places, so there is no reason why such a survey couldn't be built with international support and even use the -- well, I would have to check to see if they would -- Corriel and --- would be interested in kind of building this thing, but again if people could give me input on what kind of numbers. I am assuming the ISBT had 40 laboratories, and I think certainly we are in a numbers

scheme there that would make sense to pursue stuff like this.

DR. MOULDS: Speaking of numbers, I would like to throw this out to maybe Ghazala or Marion or Sheryl also. On the discordant samples that you are finding between serology and DNA what is the acceptable number? I know a lot of you went back and like the U negatives and the Duffy and addressed. But when we are doing our validation what should we consider as acceptable?

DR. REID: Zero.

DR. MOULDS: Yes, but sometimes the serology is wrong.

DR. REID: Well, then you have to look into them and decide what the discrepancy is due to. Otherwise how do you know if it is the tech or the test or the sample?

DR. MOULDS: Well, in our case we are using some of the --- samples, and those are 10, 12, 14 years old, and there is no way to go back and resolve them unless people want to ---.

DR. REID: We know that a lot of those were not characterized that accurately serologically.

DR. MOULDS: Right.

DR. JOHN MOULDS: There will be a lot of

scientists of supposed eminence that are going to receive some very nasty letters then.

(Laughter.)

MS. : You are mumbling.

DR. JOHN MOULDS: There will be a lot of scientists of supposed national reputation that will receive a nasty letter then, because many cells we send out we find are not holding up for their characteristics that ---.

DR. REID: The letter is fine, but it doesn't have to be nasty.

(Laughter.)

MR. : That is the only way you get their attention.

DR. YAZDANBAKHSH: So I think the bottom line is that whatever -- when we start doing the DNA typing. So if we find any discordant samples that need to be resolved as Marion said by whatever method, it is not to say the serology is always right. So we can go back and do what we did in our large-scale study. We did resolve them by sequencing and also by RFLP analysis when those assays were available.

DR. REID: We can't ignore typographical errors, too.

MS. : Yes, that is true. Sad, but it is true.

DR. MOULDS: It is true. We have already caught our lab in a Duffy.

DR. REID: It is the biggest cause of error.

DR. MOULDS: That is absolutely true. We have already seen that problem.

Closing Remarks

by Sheryl A. Kochman

MS. KOCHMAN: Well, we are getting precariously close to 2:00. I am sure many of you have planes and trains to catch, so I was going to close this out with what is next. At this point the only thing that I know is next is the transcript will come out in about two weeks. I will have to make a report of this workshop to the Blood Products Advisory Committee. They will want to know some of the questions that came up, some of the issues that were identified, and I think I can say it is not going to end there, but I don't think I can say where it is going. So we just have to all keep at.

I really want to thank everybody for coming because it has really given me a lot to think about. I hope we have given you some things to think about. One of the

issues, I should mention this also, I envision that there will probably be -- another reason I listed so many guidance documents on the list is I envision that FDA will perceive there to be a need for guidance for both manufacturers of the test kits and for the users of the kits. As I am sure you are all aware, the guidance writing process can be lengthy. One of the things has happened from time to time with guidances at CDRH is that they actually suggest to the industry that industry present the first draft of a guidance to FDA for FDA to then follow up on.

The thing you need to be aware of is because of the requirement that we follow, what are known as good guidance practices, we can freely discuss the minute issues of something as long as everything is in development, but once we decide it is time to put pen to paper and write that guidance document we can only then talk about it in general terms. We cannot talk publicly about the specifics that are going to be in it. The specifics have to wait until the draft is published, and once the draft is published it is available for public comment at that time.

So it is very helpful to get as much public comment as we can before we actual start the process, and I would ask you to be thinking along those lines so that maybe

we can start with a better guidance document to begin with and have less trouble getting it through the public comment period. But there is not a formal process for doing that though.

DR. YAZDANBAKHSH: You said during your presentation that if there is a device or a test is out in the market and the industry is presenting another test and you can show with your data that it is equivalent, then you can use the same criteria? Is that what you said? Like serology for example is licensed by FDA and the DNA analysis come in and you say, okay, you are identifying exactly the same thing but from a different way. What would you say to that?

MS. KOCHMAN: You are speaking about the substantial equivalence process. The quote, unquote, "predicate" in that process has to be either a class one or a class two device, because class three devices are either already classified as class three or if it is a new device it is actually automatically classified as class three because there is no predicate. BLA products are sort of class three devices. A class three device cannot act as a predicate for another device. So someone who wishes to pursue the 510(K) process could not say it is the same as

licensed reagents because they are not in the same class.

DR. YAZDANBAKHSH: --- you said is class one, right?

MS. KOCHMAN: Right. Blood grouping reagents are like class three devices, and 510(K) is for class one or class two. It is not as easy. It is not always as easy as it sounds. There are some cases where it is very clear cut, and I can already say that this is an area where there will be a lot of discussion on how we move forward. So I think that is it then.

(Applause.)

DR. REID: I would like to thank Sheryl for being open and putting this meeting on, organizing it and being open to our -- to hearing us. Thank you.

(The meeting adjourned at 2:05 p.m.)